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A CYTOLOGICAL EXAMINATION OF UTERINE GROWTH DURING PREGNANCY

CARLOS ALBERTO SALVATORE[1]

Clinica Ginecologica, Faculdade de Medicina[2]

UNIVERSIDADE DE SÃO PAULO, BRASIL

THE PRESENT paper aims to extend the Karyometric statistical method for the analysis of interphasic nuclear growth to a study of uterine tissues. The endocrine viewpoint was kept in mind in this paper, which deals with uterine growth in pregnancy, as also in another paper (in press) which concerns a similar cytological study of uterine growth during the estrous cycle and artificially induced estrus.

The growth response, both in size and number, of all cellular elements of the uterus during pregnancy has long been known. Gander (1930), Stieve (1929, 1932), Froböse (1934, 1935), Fabris (1935), Crandall (1938), Krichesky (1942) and others conducted their researches from the standpoint of the cell as a whole, although they do mention increase in nuclear size. It is this latter phenomenon of which the Karyometric method takes advantage.

It is believed that such a study applied to the pregnant uterus, reflecting cycles of hyperplasia and hypertrophy of the tissues, would have endocrine significance.

METHOD

Albino rats of the Institute Butantan were used in these experiments. They were killed on days 7, 13, 14, 20 or 21 of pregnancy; the uteri were fixed in Dubosq—Brasil fluid and imbedded in paraffin. The 10μ sections were stained in Haidenhain's hematoxylin and eosin.

From each representative zone 200–300 nuclei were sketched with the aid of a camera lucida at a magnification of 1890. Care was taken to select such areas in which the section passed through the long axes of the elliptical nuclei, thus avoiding errors of measurement due to inclination of the nuclear axes. In the tables the nuclei taken from the dilated parts of the uteri are

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[1] Address: Clinica Ginecologica de Hospital das Clinicas, Faculdade de Medicina da Universidade de São Paulo, Brasil.

[2] Director of Gynecological Department: Prof. José Medina.

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indicated by the letter *a* after the protocol number, the letter *b* referring to the intermediary portions. Myometrial cells of the dilated portions were measured after removal of the fetus (indicated by *c* in the tables).

Inasmuch as the Karyometric statistical method here used has not appeared in the English language, it is here described in some detail (cf. Schreiber, 1947, a, b; 1948; Schreiber and Salvatore, 1947).

Determination of Nuclear Volume. The nuclear volume of an ellipsoid was calculated after the formula:

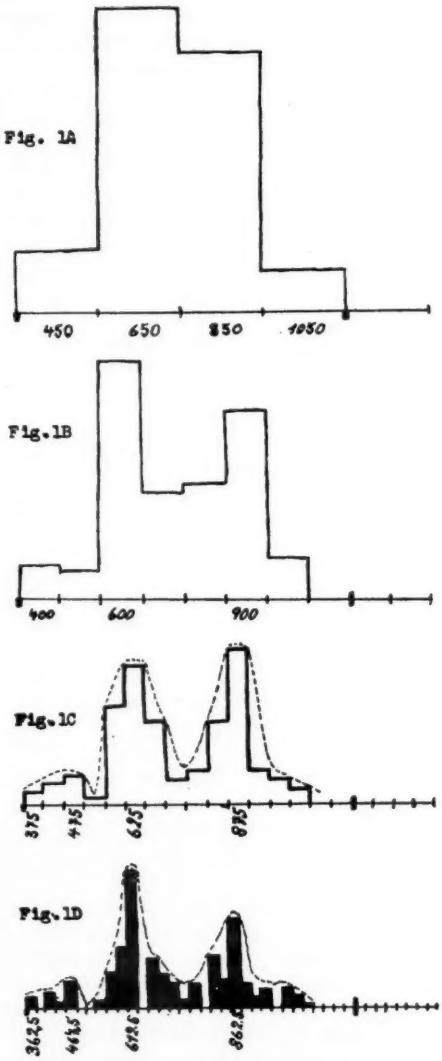
$$V = \frac{a^2 \times b}{1.91}$$

in which *a* represents the transverse, *b* the longitudinal diameter.

To illustrate, take item 39b of Table III. It was found that 3 nuclei measured 27×5 mm., and, by the above formula, volume was found to be 353.4 mm.^{3}; of 5 other nuclei, one measured 33×5 mm., volume 431.9 mm.^{3};

TABLE A. (Prot. 39b of Table III):

N° of nuclei		Long axes		Short axes	mm.^{3} (nuclear volume)	Classes	Interval of 50 mm.^{3}
3		27 mm.	×	5 mm.	353.4	between 350–399	mean 375
5	1	33	×	5	431.9	between 400–449	mean 425
	4	32	×	5	418.8	between 400–449	mean 425
6		25	×	6	471	between 450–499	mean 475
1		29	×	6	546	between 500–549	mean 525
24	9	30	×	6	565	between 550–599	mean 575
	15	31	×	6	584	between 550–599	mean 575
34	10	32	×	6	603	between 600–649	mean 625
	9	33	×	6	621	between 600–649	mean 625
	15	24	×	7	615	between 600–649	mean 625
20	12	27	×	7	692	between 650–699	mean 675
	8	26	×	7	667	between 650–699	mean 675
6		28		7	718	etc.	mean 725
8	6	23	×	8	770	....	mean 775
	2	22	×	8	750	....	mean 775
20	13	32	×	7	820	....	mean 825
	7	25	×	8	837	....	mean 825
38	22	26	×	8	871	....	mean 875
	10	34	×	7	872	....	mean 875
	6	35	×	7	897	....	mean 875
8	3	36	×	8	819	....	mean 925
	5	37	×	7	949	....	mean 925
	5	37	×	7	949	....	mean 925
6	4	38	×	7	974	....	mean 975
	2	29	×	8	971	....	mean 975
4	3	30	×	8	1005	....	mean 1025
	1	31	×	8	1038	....	mean 1025



FIGS. 1A–D. Histograms illustrating the result of graphing the data for case 39b (Table III) according to different class intervals: 1A, 200 mm.^{3}; 1B, 100 mm.^{3}; 1C, 50 mm.^{3} and 1D, 25 mm.^{3}. The histograms are drawn on different scales.

4 nuclei, 32×5 mm., had a calculated volume of 418.8 mm.,³ and so on, as shown in Table A. Absolute values can be calculated if one wishes; one mm. corresponding to 0.53 micron; but this has not been considered necessary, as the study is one of relative volume variations.

Statistical Elaboration of Results. In this Arkin and Colton's Outline of Statistical Methods, 4th edition, Barnes and Noble, Inc., New York, 1942, served as a guide.

The values found (nuclear volumes) were grouped in frequency classes in ascending series of sizes of nuclei, as may be seen in Table III, for example. The class interval here chosen, for reasons to be discussed below, is 50 mm.³ Thus all nuclei having volumes between 400 and 449 were collected under one class represented by the average value 425; all between 450 and 499 were collected under the average value 475 and so on (Table A).

TABLE B. (Prot. 39b of Table III):

Classes of 50 mm.³ (or 0.50):														
375	425	475	525	575	625	675	625	775	825	875	925	975	1,025	etc.
3	5	*6*	1	24	*34*	20	6	8	20	*38*	6	4		Frequency (Fig. 1C)
Classes of 100 mm.³:														
400		500		600		700		800		900		1,000		etc.
8		7		*58*		26		28		*46*		10		Frequency (Fig. 1B)
Classes of 200 mm.³:														
450				650				850				1,050		etc.
15				*84*				74				10		Frequency (Fig. 1A)

Class intervals of 25 mm.,³ 100 mm.³ and 200 mm.³ might have been used as shown for protocol 39b in Table B. The superiority of the 50 mm.³ interval for Table III will be apparent from a persual of the frequency histograms shown in Figure 1A to 1D.

Figure 1A demonstrates the fact that with a class interval of 200 mm.³ only one modal class (at 650) emerges. With an interval of 100 mm.³ three modal classes are noted, namely at 400, 600 and 900 mm.³ (Fig. 1B). Greater neatness of the three modal classes (at 475, 625 and 875) is seen when the class interval of 50 mm.³ is utilized, as in Figure 1C, which represents case 39b of Table III (cf. Table B). The 50 mm.³ class interval has, therefore, been employed for the nuclear volumes of muscle cells (Table III). For glandular and endometrial cells, whose volumes are greater, by the same method of analysis, the 100 mm.³ class interval was chosen.

It is apparent that the probabilities of mistake is reduced in proportion to the reduction in the class interval (Arkin and Colton). Thus the 25 mm.³ interval (Figure 1D) gives even a better curve than the 50 mm.³ interval but not enough better to warrant the space necessary for presenting double the number of classes. Fifty mm.³ would, therefore, seem both accurate and practicable.

Further analysis of our data is presented in Figure 2, which reports average histograms made up of the arithmetrical mean of the nuclear volumes of day 7, 13, 14 and 20, 21, respectively. The calculations were made from Table III as follows:

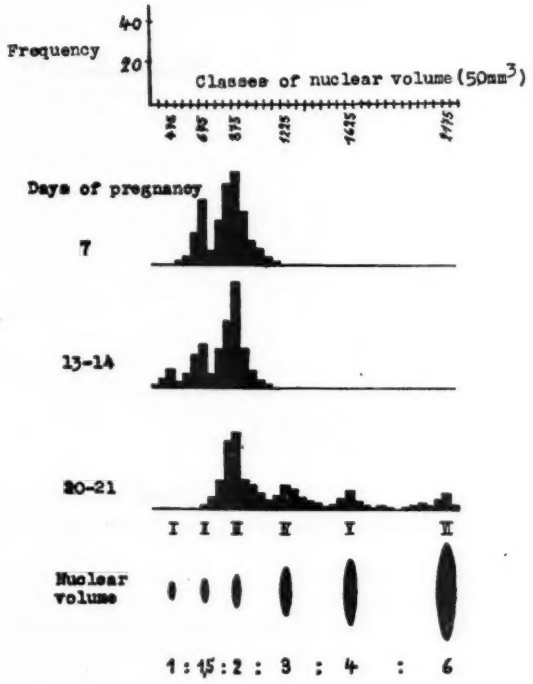


FIG. 2. Average histograms of nuclear volumes of muscle cells at three stages of pregnancy (see Table III); on the ordinates, nuclear volume frequencies; on the abscissas, class intervals. Below, schematic diagrams of nuclear sizes.

The nuclei of each class pertaining to the same time of gestation were added and the sum divided by the number of cases involved. Thus in the 525 mm.³ class, there is one nucleus of case 36a and two of 36b, a total of 3 nuclei in two cases, an average of 1.5. In class 575 the average calculates to 4 (12 nuclei in three cases).

Because of lack of space no table has been presented of these calculations —the histograms of Figure 2 must suffice to show the trends. Under the histograms, the nuclear volumes are represented schematically.

Determination of Modal Value. In these calculations of modal values, Arkin and Colton's formula (p. 23) has been followed:

$$M = L + \left(\frac{F}{F+f} \times i\right).$$

To illustrate with case 39b of Table III which reveals 3 maxima of frequency (i.e. 3 modal classes): 475, 635 and 875 mm.³ (cf. Fig. 1C): In this case the first modal class is that of 475 mm.³ (Figure 3).

L = the lower limit of the modal class, i.e. that between 425 and 475, or 450 mm.³

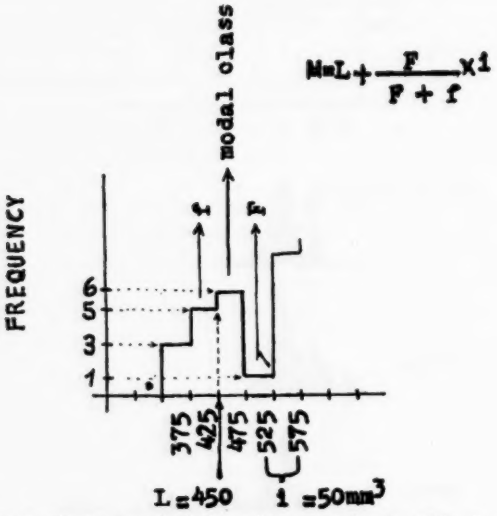


FIG. 3. To illustrate calculation of the modal value (M). See test.

F = the frequency of the class above the modal class; in our example it is represented by one nucleus ($F = 1$).

f = the frequency of the class below the modal class, namely 5($f = 5$).

i = the class interval, which in the case of the myometrium (Table III) is 50 mm.[3]

Substituting:

$$M = 450 + \left(\frac{1}{1+5} \times 50\right) = 458$$

458 therefore, represents the modal value of the first class of maximal frequency for the individual case selected, namely 39b of Table III.

The second modal value is calculated thus:

$$M = 600 + \left(\frac{20}{20+24} \times 50\right) = 622$$

For case 39b we find modal values 458, 622 and 864.

$864 \div 458 = 1.88$ or approximately 2

$864 \div 622 = 1.38$ or approximately 1.5

Persuing the calculations further we arrive at figures closely approximating the theoretical numbers of the progression: 1: 1.5: 2: 3: 4: 6 etc.

For the sake of brevity only Tables I, II and III are presented. These show the frequencies of the calculated nuclear volumes and the calculated modal values, the fundamental values in this research.

The mitotic indices and the degree of infiltration of leucocytes are also presented in the tables.

The reality of the modal values, in the purely statistical sense, is proven not only by the constancy of the analyzed cases but also by comparison of

TABLE 1. UTERINE LINING EPITHELIUM

N. of protocol	Days of pregnancy	Modal value				Nuclear volume																	N. of nuclei	Mitotic index	Leukocytic infiltration
		I	II	III	IV	350	450	550	650	750	850	950	1050	1150	1250	1350	1450	1550	1650	1750	1850	1950	2050			
15 a	7		1061	1432							4	10	*20*	16	20	42	*60*	20	5					197	—	—
15 b	7			1437								5	10	10	24	38	*55*	23	7					172	—	—
36 a	7		1061	1361						2	2	5	*10*	8	25	*45*	40	20	9	1				167	—	—
36 b	7			1430							4	4	8	10	30	35	*50*	15	8	2				166	—	—
38 a	13	657	1050	1431	1933	2	3	9	*23*	12	10	10	*19*	10	13	31	*40*	14	10	2	2	*6*	1	217	4,3	+
38 b	13	654	1037	1430	1900		2	5	*15*	6	8	10	*25*	6	14	28	*35*	12	7	5	1	*3*		182	3,1	+
39 a	14	665	1047	1438			4	7	*18*	13	13	10	*18*	9	21	30	*42*	19	8	3				215	3,3	+
39 b	14	636	1036	1371			5	21	*38*	12	5	12	*15*	7	16	*45*	40	12	4	1				233	2,4	+
17 a	20			1454								1	5	14	13	27	*60*	32	9	1				162	—	+
17 b	20			1440									2	8	24	42	*76*	28	5	2				187	—	+
2 a	20			1444	1937									5	12	30	*74*	24	24	13	5	*10*	3	200	—	+
2 b	20			1443	1966									2	10	26	*62*	20	13	8	1	*5*	2	149	1,7	+
22 a	21			1447	1950								1	3	12	30	*82*	27	11	5	1	*3*	1	176	3,9	++
22 b	21			1423	1960									2	8	32	*40*	10	3	3	4	*8*	6	116	5,7	++

TABLE 2. GLANDS

N. of protocol	Days of pregnancy	Modal value				Nuclear volume																				N. of nuclei	Mitotic index	Leukocytic infiltration
		I	II	III	IV	250	350	450	550	650	750	805	950	1050	1150	1250	1350	1450	1550	1650	1750	1850	1950	2050	2150	2250			
15 a	7			1363										*5*	12	35	*68*	60	29	9							218	—	−
15 b	7		1052	1422									8	*12*	9	28	47	*50*	14	8							176	—	−
36 a	7		1100	1445							2			*10*	6	27	33	*64*	27	*5*							174	—	−
36 b	7		1127	1452								1	8	16	*20*	6	27	50	30	10							168	—	−
38 a	13	680	980	1448					1	*5*	4	5	*25*	20	8	5	30	*40*	28	4							175	—	+
38 b	13	680	960	1425					2	*14*	8	6	*10*	9	7	15	45	*47*	15	2							180	—	+
39 a	14	688	1026	1424				1	2	*16*	15	14	28	*43*	10	7	25	*26*	8									196	—	+
39 b	14	960	988	1362				2	2	*19*	18	3	*25*	24	12	21	*36*	35	15	4								216	—	+
17 a	20			1430	1833								5	10	28	35	62	*71*	27	7	2	*4*	1				252	0.6	++
17 b	20			1360									3	6	16	40	*69*	61	16	10	5						226	—	+
2 a	20			1435	2066									1	22	36	41	*100*	23	13	8	6	1	*5*	2		258	0.4	++
2 b	20			1431	1971								2	2	32	36	80	*94*	36	14	5	2	*7*	5			315	—	++
22 a	21			1425	1014										10	32	58	*80*	20	8	5	1	6	*10*	1		231	4.7	++
22 b	21			1428	1987										8	30	45	*60*	18	*5*	—	1	*8*	7	2		184	5.2	++

TABLE 3.

N. of protocol	Days of pregnancy	Modal value						Nuclear														
		I	II	III	IV	V	VI	375	425	475	525	575	625	675	725	775	825	875	925	975	1025	1075	1125	1175
15 a	7		655	870								2	8	*15*	1	18	35	*50*	25	16	13	3	2	
15 b	7		678	860									5	*16*	7	8	45	*60*	12	8	2			
36 a	7		666	829							1	4	25	*50*	12	28	*46*	40	27	7	5	3	2	
36 b	7		616	864							2	6	*14*	3	2	10	26	*53*	11	5	2			
38 a	13		662	866						3	4	6	19	*28*	6	10	27	*51*	13	6	2			
38 b	13		668	876								1	5	*14*	3	7	16	*33*	18	8	4	2		
39 a	14	460	626	868				1	8	*16*	2	13	*16*	15	10	29	39	*55*	22	12	4	3	2	
39 b	14	458	622	864				3	5	*6*	1	24	*34*	20	6	8	20	*38*	8	6	4			
17 a	20			829	1183									2	6	30	*58*	44	12	10	3	1	1	*4*
17 b	20			836	1183	1583								3	8	30	*100*	78	25	14	6	2	4	*12*
2 a	20			864	1216	1590	2133							5	8	18	42	*45*	17	15	10	5	6	10
2 b	20			883	1233	1618	2131									8	10	*25*	20	9	9	6	5	8
2 c	20			836	1222	1545	2162								5	6	*20*	16	12	3	9	2	3	15
22 a	21			870	1167	1576								2	4	7	18	*40*	12	11	11	13	15	*26*
22 b	21			833	1163	1580									2	20	*44*	40	32	15	16	8	22	*27*

all the maxima of frequencies pointed out in the tables; these maxima show a perfect correspondence among those values in the different stages studied.

It may further be pointed out to the reader that a modal class which may be called "Secondary" because it is made up of few nuclei in one phase of pregnancy, may become a "principal" modal class in another phase. This fact demonstrates the biological reality of the modal value, in correlation with the rhythmic growth of the studied material.

RESULTS

If we compare the 3 charts, the first thing that strikes us is that the variations of nuclear volumes in all tissues examined are similar, and that they take place simultaneously. Furthermore, the increase of the nuclear volume is not steady, but rhythmic, the modal values of which are proportional to a basic modal value. Besides this, the variations of nuclear volumes are exactly the same in non-dilated intermediary as well as in the dilated parts.

In the first part of these researches (1948) we recorded the variations of nuclear volumes during the estrous cycle and artificially induced estrus and showed that the lowest modal values found were 704 in the epithelium, 664 in the glands and 424 in the myometrium, which values we take to be our *basic values* (mode I).

We next observed on the 7th day of pregnancy a first mode of 1061 in the epithelium, 655–666 (a) and 678–616 (b) in the myometrium. All these figures corresponding roughly to value 1.5 of the above mentioned basic nuclear volume; the same was found during diestrum, metestrum and in castrated animals. This modal value, which is 1.5 times the basic modal value, we called mode II. The highest frequency of nuclear volumes was found in modal value 1432–1361 (a), 1437–1430 (b) in the epithelium and 870–829 (a), 829–864

MYOMETRIUM

volume																						N. of nuclei	Mitotic index	Leukocytic infiltration
1225	1275	1325	1375	1425	1475	1525	1575	1625	1675	1725	1775	1825	1875	1925	1975	2025	2075	2125	2175	2225				
																						188	0.5	–
																						163	–	–
																						250	0.4	–
																						134	–	–
															•							175	1.1	+
																						111	–	+
																						247	0.4	+
																						183	–	+
2																						173	–	+
8	2	1	1			1	*10*	2														307	–	++
12	5	2		1		1	*12*	4	1		2	1		1	1	3	1	*5*	2			235	–	++
19	16	9	7	6	2	2	18	*39*	11	20	6			3	5	3	6	*12*	10	2		296	0.9	++
24	12	8	6	2	1	*12*	9	4	1		2	1			1	7	2	12	*21*	4		220	–	++
8	3		1		3	6	*12*	7	2													201	–	++
8	6	2	1		1	2	*5*	3		1				3		1						259	–	++

(b) in the myometrium which correspond roughly to twice the basic value (mode III).

The glandular epithelium shows variations similar to those of the lining epithelium. There is neither infiltration of leucocytes, nor mitoses: only in the myometrium mitoses were some observed (mitotic index: 0.5 and 0.4%: *a*).

On the 13th and 14th day of pregnancy there appeared both in the endometrium and the myometrium, basic modal values (mode I) followed by mode II and also the mode which represents the highest frequency on nuclei, of twice the basic volume (mode III). A mode IV, at 3 times the basic value was observed in the glandular endometrium.

At this stage of pregnancy we found also histological changes similar to those of estrus, i.e., there is a typical infiltration of leucocytes and mitotic forms are present in the glands and superficial endometrium, as well as, in the myometrium (mitotic index 1.1 and 0.4%). On the 20th and 21st day of pregnancy the first mode observed represents values of 1454–1444 etc. in the endometrium, and 829–864 etc. in the myometrium which equal mode III (twice the basic volume), while there is a complete absence of lower values. On the other hand, besides mode IV that we found in both tissues, we found in the myometrium nuclear volumes of a modal value 4 times the basic value (mode V) (average 1592) and of a modal value 6 times the basic value (mode VI) (average 2188).

If we look at the histograms Figure 2 showing the myometrium, (the same holds for the endometrium) we find the variations of the nuclear volumes to be strictly rhythmic. We shall later refer to the ratios between the stages of the rhythmic growth.

On the 20th and 21st day of pregnancy, much infiltration of

leucocytes was seen in all uterine tissues: mitoses were found in the glands and superficial epithelium as well as in the myometrium.

DISCUSSION

The increase in size of the uterus during pregnancy results, as is now well known, chiefly from hypertrophy and hyperplasia depending on hormonal function and on the mechanical distention of the uterus, caused by the foetus it encloses.

We shall discuss separately the phenomena of hypertrophy and hyperplasia of the uterine elements and compare our data with those of the existing literature on the subject.

Hypertrophy: The increase in size of each individual uterine cell is generally admitted to be the main factor of uterine growth. Koelliker (1840), quoted by Crandall (1938), held there is an enlragement of muscle cells (hypertrophy) during pregnancy, as well as, hyperplasia. Hammond (1935), Reynolds (1937), Markee and Hinsey (1935) and Miyasaki (1938) observed an increasing thickness of the myometrium during gestation. Markee; Wills and Hinsey (1936) also observed an increase in size of the endometrium and of the connective tissue. Krichesky (1942), on the basis of his experiments with intraocular transplantation of uterine parts, held that individual hypertrophy of muscle fibres and interstitial swellings in the muscles, contribute to the enlargement of the myometrium. He further states that the superficial endometrium is also subject to hypertrophy during the first days of pregnancy. Crandall (1938) measured the volume of the muscle cells and found that the hypertrophy of these cells corresponds to $\frac{1}{5}$ or $\frac{1}{6}$ of the total increase of uterine volume.

From the cytological point of view, the variations of cellular volume during pregnancy were established almost exclusively with regard to muscle tissues. Stieve (1927, '29b) working with the human uterus, and Gander (1930) working on mice, measured not only the enlarged muscle cells but also their nuclei and proved that they also show variations in size.

Froböse (1932, '34, '35), examining uteri of rabbits and albino rats in various stages of pregnancy, observed that the cells as well as their nuclei grew steadily, reaching maximum size during delivery. He also observed (1932) that the muscle cells of the non-dilated uterine parts, did not attain to the same size but that their nuclei did, which means that the increase in nuclear volume was the same in all parts of the uterus.

Fabris (1935) examining uteri of cows, of pregnant women and cases of extrauterine pregnancy, obtained results similar to Froböse's both as to muscle cells and their nuclei. These two authors as well as Knaus (1929) also attribute these changes in nuclear size to hormonal agents.

As can be seen on the tables and on Figure 2 the results of our

research do not only prove the presence of nuclear hypertrophy (endometrium and myometrium) but they also show that this is due to hormonal agents, since the changes in the nuclear volume are exactly the same, and are simultaneous, in the dilated parts enclosing the foetuses and in non-dilated intermediary parts. Besides, the hormonal agents (estrogens) must be chiefly responsible for the hypertrophy, since, in a previous publication (1948, 4Fa), we have shown that estrone injected into castrated rats induced the growth of the nucleus to twice its size, which is equal to the nuclear volume found during the normal estrous cycle and during pregnancy.

That the real origin of the nuclear growth during pregnancy appears to be at best unknown, is illustrated by the fact that Keiffer (1928, '29) attributed uterine enlargement (human and guinea pig) to the imbibition of cellular water. The author also records that the increase in size to all muscle cells and the increase in number of myofibrillas is connected with the extensibility and contractibility of the tissues. He also holds these changes to be due to hormonal agents.

Our karyometric research however on endometrium and myometrium during the estrous cycle and estrus artificially induced by oestrone (1948, 1947a), as well as the results recorded in this paper, prove that the increase of the nuclear volume is not steady but rhythmic, the modal values of "leap" being in proportion to the basic value at the rate of 1:2:4:8 etc.

Modern karyometric research work on the interphasic growth of the nucleus shows a very evident relation between the nuclear volume and the number of chromosomes and chromonemata. Fabris (1935) already had said "It is interesting that it should be the nucleus and not the cytoplasma that starts hypertrophy." In 1935 this author put forward the following hypothesis: "It seems possible that the increase in size of the nucleus is related to a prolific cellular capacity."

From the numerous karyometric researches we may conclude that the *rhythmic growth of the nucleus* is related to the *multiplication of the nuclear "genome"* or *genetic elements* during the interphasic growth of the nucleus. Consequently, we must admit that we call hypertrophy is, at least as regards to the nucleus, due to multiplication of the nuclear genome.

This interphasic growth of the nucleus may show an intermediary value of 1.5 times the basic volume which has been discussed in many of Schreiber's researches and in a previous publication in collaboration with the author (1947a).

We found that on the 7th day of pregnancy (Figure 2) the greatest number of nuclei show twice their basic volume (mode III) and some show 1.5 times their basic volume, which points to a pause in interphasic growth. On the 13th and 14th day of pregnancy small nuclei are present, a phenomenon which we shall discuss later when dealing with hyperplasia. On the 20th and 21st day of gestation the

results obtained are very interesting, especially as to the myometrium, since, besides the absence of small nuclei, the histogram shows that several nuclei keep on growing and attain three, four and six times their primitive size (mode IV, V and VI).

In the endometric tissues the nuclei are large, but generally they do not exceed twice their primitive size (mode III).

Summarising, our karyometric researches on uterine tissues show that hypertrophy during pregnancy depends on *phenomena connected with the multiplying mechanism of the cells*. The real nature of hypertrophy of nuclei, is interphasic growth, both when mitotic division follows, and probably endomitotic growth when the nuclei exceed twice their primitive size without dividing. For a further discussion on the mechanism and significance of this rhythmic growth of the cell nucleus the reader must refer to Schreiber (1943, '46).

Hyperplasia: A considerable number of authors, among them Vilian (1849), Heschl (1852), Luska (1864), Veit (1885), Sanger (1887), Nagel (1896), Morales, Hoekl and Meyer all quoted by Froböse (1932), believe that hyperplasia is the principal factor of uterine growth. Amati (1992), quoted by Fabris (1935), observed hyperplasia in the myometrium of rabbits brought about by mitotic division of the muscle cells during the first days of pregnancy. Cattani and Pestalozza (quoted by Fabris, 1935) and Grynfelt (1924–'25) found mitoses in the muscle cells of the human at their highest frequency during the first months of gestation. These facts were confirmed by Gander (1930) who observed hyperplasia in the uterine muscle of pregnant rats and mice resulting only from mitoses of pré-existent muscular cells. The same was held by Froböse (1932) who, noted in rabbits that "the muscle cells increased in number by mitotic division."

Fischer-Wassels and Büngeler (1931) found mitoses of the muscle cells in the uterus of baby mice after treatment with urine from pregnant woman, and Allen, Smith and Gardner (1937) observed the same in normal rats and mice treated with anterior pituitary extract and pregnant-mare serum. Crandall (1938) employing the colchicine method found abundant mitoses in the circular muscle of the uterus of rabbit, right at the beginning of pregnancy. Generally, authors admit that the generation of new cells in the uterus, both in the myometrium and endometrium, is limited to beginning of gestation, although Froböse observed that it may occur during the whole period of gestation.

Our own observation also shows, as may be seen on the histograms, that hyperplasia is present on the 13th and 14th day, when even the histologic picture is similar to that of estrus. The presence of small nuclei (mode I) besides the presence of numerous mitoses in the endometrium and rare mitoses in the myometrium (mitotic index 1.1 and 0.4%) indicate hyperplasia, since it is highly probable that the small nuclei are the product of division of large (mode III).

On the other hand, the fact that we find on the 13th and 14th day of pregnancy a picture similar to that of estrus confirms the experiments made by Swezy and Evans in 1930. These authors relate that the graafian follicles in rats show growth and regression at a rate approximately corresponding to the rhythm of the estrous cycle i.e. on the 5th, 10th, 14th and 18th day of gestation. It's quite possible that the values shown on our histograms of the 13th and 14th days of pregnancy of the rat, will also be found on the other days indicated by Swezi and Evans. Moreover, the histograms of the 7th day are very similar to those of the proestrum (Salvatore, 1948). If this is so,

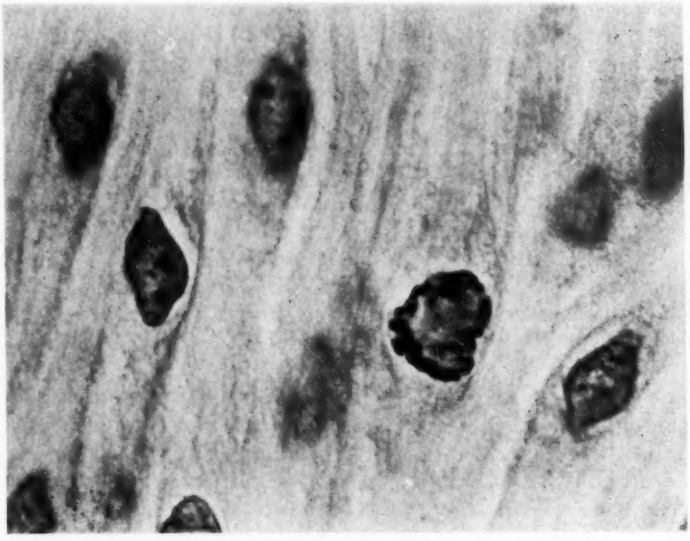


FIG. 4. Typical mitoses (prophase) of muscle cells on day 20 of pregnancy (magnification ×1480).

mitoses will be found during the several stages of pregnancy (in rats) including the last days in the endometrium as well as in the myometrium. On Figure 4 are shown typical mitoses (prophase) of muscle cells on the 20th day of pregnancy.

Furthermore, the partial development of the follicle in the ovaries as well as the presence of mitotic forms in the uterus would be dependent on the threshold of the circulating hormone. In our foregoing publication (1948), we said that one fact struck us as very significant: after discontinuing the oestrone treatment of castrated animals there appeared numerous mitoses, which might indicate that the decline of the estrogenic threshold is the factor which determinates the mitoses in uterine tissues. Crandall holds however that the hyperplasia in the myometrium is due to a combined effect of estrin end progestin.

There is, however, another factor that may determine the presence of hyperplasia: it is the mechanical distention of the uterus as proved by the experiments of Reynolds and Allen (1937), Reynolds and Kamminester (1937a, 37b) and Allen, Smith and Reynolds (1937) who employed the colchicine method, found typical mitoses in the muscular cells of castrated rabbits and in the castrated rabbits after progestin had been injected. It must, however, be kept in mind, that hyperplasia without hypertrophy—and vice versa—has never been found, not even in those objects of examinations obtained by mechanical distention, which proves the inter-relation of these two phenomena.

As was shown with reference to the estrous cycle and to artificially induced estrus in our previous publications (1948) and may be read off our present histograms, *hypertrophy* always precedes the appearance of mitotic forms, which proves that nuclear hypertrophy is actually the *interphasic growth of the nucleus*. Soon after this first phase, during physiological estrus, after discontinuing estrone treatment, and on the 13th and 14th day of gestation (very likely also in other stages), mitotic division sets in, the evidence for which, on our histograms, is the presence of small nuclei and mitotic forms. It is probable that during pregnancy only a part of the cells is subject to division, while the other either stops growing at twice their original volume or continues its interphasic growth, attaining to large volumes as is shown on the histograms corresponding to the 20th and 21st day of pregnancy.

The absence of small nuclei (mode I) on the 20th and 21st day of pregnancy, and the presence of such on the 13th and 14th day, together with the decrease in number of large nuclei (mode III) and accompanied by mitoses, seem to show that the new cells of the myometrium have their origin in pré-existent muscular cells of the uterus. In our opinion evidence for this, is to be found on the histograms corresponding to physiological and artificially induced estrus, completing our previous paper (1948).

On this point there is difference of opinion. Keiffer (1928, 1929) would have muscle cells originate from fibroblasts, Joachimovits (1928, 1929) from special forms of histiocytes. Stieve (1929a, 1929b, 1932) agrees more or less with both. Froböse (1935) contends for a combined original from connective tissue and muscle cells. Crandall (1938) and Corner (quoted by Reynolds, 1939) agree that a transformation of connective tissue to muscle cells is possible.

With these Fischer-Wassels and Büngeler (1931) disagree on the basis of their trypan blue, for muscle cells never contain granules of the dye as do connective tissue cells.

On the basis of the author's previous work on the estrous cycle of the rat and the present study of the pregnant rat, the most reasonable interpretation of the statistical findings with the karyometric tech-

nique employed is that new muscle cells of the uterus arise from pré-existing muscle cells.

CONCLUSIONS

The variations of nuclear volumes in the superficially and glandular endometrium and in the myometrium during pregnancy are similar and take place simultaneously according to the stage of pregnancy.

The increase in volume of the nuclei is exactly the same in the enlarged uterine parts, enclosing foetus, as in the non-dilated intermediary parts.

The increase in the nuclear volume during pregnancy is not steady, but rhythmic, i.e. it proceeds in leaps. The duplication of the nuclear size between 2 steps, indicates with probability the genetic nature of that growth.

The uterine growth is mainly consequence of one phenomenon, i.e. the multiplication of the nuclear genome. Hypertrophy and hyperplasia are manifestations of this process.

a) Hypertrophy of the nuclei is mainly interphasic growth, after which mitotic division take place; probably it may also be "endomitotic growth" when the nuclei keep growing and attain three, four and six times their primitive size even though they do not divide. The genetic significance of the rhythmic growth has been discussed in the papers of Schreiber.

b) Hyperplasia is the final of interphasic growth of several nuclei.

In the myometrium hyperplasia very likely takes place by means of division of pré-existent muscular cells and not by means of transformation of connective tissue cells into muscular cells.

On the 13th and 14th day of pregnancy the cells multiply in cycles similar to those that may be observed during estrus.

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REFERENCES

Allen, E., G. M. Smith, and S. R. M. Reynolds: *Proc. Soc. Exper. Biol. & Med.* 37: 257. 1937.
Allen, E., G. M. Smith, and W. V. Gardner: *Anat. Rec.* 67: 3. 1937.
Biesele, J., H. Poyner, and T. Painter: *The Univ. Texas Public.* nov. 15. 1942.
Crandall, W. D.: *Anat. Rec.* 73: 195. 1938.
Fabris, M.: *Atti della Soc. Med. Chirurg. di Padova.* marzo. 1935.
Fischer-Wassels, B., and W. Büngeler: *Verhabdl. d. Deutsch. Pathol. Gesellsch.* 26. S. 1931.
Froböse, H.: *Ztschr. f. mikr. anat. Forsch.* 30: 295. 1932.
Froböse, H.: *Ztschr. f. Geburtsh. u. Gynäk.* 109: 31. 1934.
Froböse, H.: *Ztschr. f. mikr. anat. Forsch.* 37: 17. 1935.
Gander, G.: *Zeit. f. d. ges. exp. Med.* Bd. 72: 44. 1930.
Grynfelt, E.: *Bull. Soc. de sc Méd. et. de Biol. de Montpellier.* 6: 441. 1924–25.
Hammond, J.: *Trans. Dynamics of Development.* 10: 93. 1935.
Joachimovits, R.: *Biol. General.* Bd. 4: 447. 1928.

JOACHIMOVITS, R.: *Arch. f. Gynäk.* 135: 536. 1929.
KEIFFER, H.: *Bull. Acad. de med. Paris.* 99: 529. 1928.
KEIFFER, H.: *Bruxelles Med.* 9: 1. 1929.
KNAUS, H.: *München med Wchnschr.* 76: 40. 1929.
KRICHESKY, B.: *Anat. Rec.* 82: 551. 1942.
MARKEE, J. E., AND J. C. HINSEY: *Anat. Rec.* 61: 311. 1935.
MARKEE, J. E., W. M. WELLS, AND J. C. HINSEY: *Anat. Rec.* 64: 221. 1936.
MIYAZAKI, T.: *Fukuoka acta med.* 39.: 1827. 1934.
ORVILLE, L. B., AND M. O. OVERHOLSER: *Anat. Rec.* 70: 401. 1938.
REYNOLDS, S. R. M.: *Am. J. Obst. & Gynec.* 33: 968. 1937.
REYNOLDS, S. R. M.: Physiology of the Uterus. pg. 127. 1939. Hoeber New York.
REYNOLDS, S. R. M., AND W. M. ALLEN: *Anat. Rec.* 69: 481. 1937.
REYNOLDS, S. R. M., AND S. KAMINESTER: *Anat. Rec.* 69: 281. 1937a.
REYNOLDS, S. R. M., AND S. KAMINESTER: *Proc. Soc. Exper. Biol. & Med.* 36: 257. 1937b.
SALVATORE, C. A.: *Rev. Bras. Biol.* (in press). Dezembro. 1948.
SALVATORE, C. A., AND G. SCHREIBER: *Mem. Inst. Butantan.* 20: 39. 1947a.
SALVATORE, C. A., AND G. SCHREIBER: *Mem. Inst. Butantan.* 20: 335. 1947b.
SCHREIBER, G.: *Rev. de Agric.* (Piracicaba). 18: 453. 1943.
SCHREIBER, G.: *Rev. Bras. de Biol.* 6: 199. 1946.
SCHREIBER, G., AND C. A. SALVATORE: *Boll della Soc. Ital. di Biol. Sper.* 23: 347. 1947.
SCHREIBER, G., AND M. R. SCHREIBER: *Bol. Fac. Cienc. Letr. U. S. Paulo.* 22: 234. 1941.
STIEVE, H.: *Verhandl. Anat. Ges.* 36: 51. 1927.
STIEVE, H. *Ztschr. f. mikr-anat. Forsch.* 17: 371. 1929a.
STIEVE, H.: *Verhandl. Anat. Ges.* 38: 27. 1929b.
STIEVE, H.: *Zentralbl. f. Gynäk.* 56: 1442. 1932.
SWEZY, O., AND H. M. EVANS: *Science.* 71: 46. 1930.
VALLE, G.: *Ginecologia.* 1: 875. 1935.

THE USE OF STILBESTROL INHIBITED MALES AS TEST ANIMALS FOR GONADOTROPHIC HORMONES

A. V. NALBANDOV AND G. J. BAUM
From the Department of Animal Science
UNIVERSITY OF ILLINOIS, URBANA, ILLINOIS

THAT ESTROGENIC substances have an inhibiting effect on the gonadotrophic activity of the pituitary of mammals has been amply demonstrated in endocrine literature. The use of stilbestrol as a "fattening" hormone in chickens has shown that a pronounced modification of the rate of function of the pituitary may be accomplished. Implants of as little as 15 mgs. of stilbestrol into males will cause complete regression of the testes and subsequently the comb, while larger doses will lead to a cessation of growth and will induce conditions in some instances resembling surgical hypophysectomy (Nalbandov & Card, 1943). These observations led to the idea that the physiological inhibition of the pituitary by estrogen may be used in the laboratory in place of surgical hypophysectomy. The advantage of the former lies in the fact that it is possible to make implants into large numbers of animals in a very short time, that mortality is as low as that of controls and that physiologically hypophysectomized males may be restored to normal, if that should be desired, by removing the pellets of stilbestrol. Unlike hypophysectomized animals, physiologically inhibited males can be kept under the same environmental conditions as untreated controls, the mortality being not any greater than that of the controls. It is also possible to produce "degrees" of physiological hypophysectomy by varying the dosage of estrogen given.

The disadvantages of this method are that occasionally a few of the males (1–2%) do not respond to the dose of stilbestrol given. In some males, a very small percentage, the implants become incapsulated and the pituitary may begin to function subnormally or even normally at an inopportune time in the experiment. Such cases are, as a rule, easily detected. In spite of these drawbacks, which are not any greater than the possibility of incomplete hypophysectomy or the danger from postoperative mortality, it appears that physiological hypophysectomy of males has definite advantages as a substitute for surgical hypophysectomy.

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ANIMALS AND TECHNIQUES

White male rats of the Sprague-Dawley strain weighing around 40 grams were used. In one experiment a pellet containing 15 mg. of diethylstilbestrol was implanted subcutaneously into each rat while in another experiment two such pellets were used. The implant can be made with or without light ether anesthesia, the slit in the skin being made in the region of the posterior dorsal third of the body. The pellet is then manipulated until it is lodged between the shoulder blades. It is recommended that reasonably clean techniques be used during this operation and that the pellet be dipped in alcohol prior to implantation. These precautions reduce the danger of infection and abcess formation which contribute to the undesirable incapsulation of pellets.

In the chicken the technique is similar except that here the implant is made in an area which is free from feather tracts, usually along the breast. This makes it possible to see the pellet through the skin and to check the birds periodically for the condition of the implant. In all our experiments, White Leghorn chickens were used. These are cheaper than are the males of the heavy breeds and are found to be much more sensitive to exogenous and endogenous hormones. The ages of the birds used varied from 20 to 200 days. In the experiments discussed here, the males weighed on the average 750 grams at the start of the experiment and were about 70 days old.

To test the response of stilbestrol implanted males to gonadotrophic hormones, PMS (Gonadogen) and purified preparations of LH and FSH were used. The PMS was injected at the rate of 25 Cartland-Nelson units per male in 8 days, while the FSH or LH preparations were injected at the rate of 5 grams equivalent (in terms of acetone dried sheep pituitary powder) per male in 8 days.

On all rats the following measurements were obtained at the time of autopsy: body weights, testes, seminal vesicles, adrenals, thyroids, and pituitary weights. In chickens, in addition to the above measurements comb size (the product of length and height) was used as the index of the rate of androgen secretion.

Two experiments using rats were done. In the first, the implants were made when the males were 21 days old. Each 7-day period after the day of implantation 5 treated and 4 untreated control rats were killed and the organ weights obtained. Thus a group of 5 males each was killed when it had been under the influence of stilbestrol for 7, 14, 21, and 28 days, respectively. The rats in each group were compared with untreated animals of the same sex and age.

In the second experiment two 15 mg. pellets of stilbestrol were implanted into each male and a period of 21 days was allowed to elapse before the first sample of organs was taken. At the end of the 21-day implantation period, some of the estrogenized rats were started on injections of pregnant mare serum (PMS—Gonadogen). Again in this experiment autopsy was performed every 7 days so that successive groups of 3 rats each had been implanted 21, 28, 35, 42, 49, and 56 days, respectively. Other groups (5 rats in each) had been implanted the same number of days as the above groups but in addition had been injected with PMS for 7, 14, 21, 28, and 35 days. A third series of groups of 3 rats each had received neither hormone treatment and served as normal controls.

TABLE 1. COMPARISON OF THE EFFECTS OF STILBESTROL ON THE ORGAN WEIGHTS OF RATS AND CHICKENS

Animals used	Length of experimental period (days)	Hormone Treatment	Testes				Seminal Vesicles or Comb Area				Average weight of organ during experimental period (% of body weight)	
			Start		Finish		Start		Finish			
			Weight (gms.)	% of body weight	Weight (gms.)	% of body weight	Weight or area (gms./cm.²)	% of body weight	Weight or area (gms./cm.²)	% of body weight	Testes	Seminal vesicles/comb
Male Chickens	35	Control	0.22	0.02	2.33*	0.16	25.99	—	62.38*	—	0.10*	—
		Stilbestrol	0.21	0.02	0.48*	0.003	13.39	—	14.50*	—	0.01*	—
Male Rats	35	Control	0.69	0.90	2.72*	1.34	0.06	0.07	0.45*	0.22	1.03*	1.20
		Stilbestrol (15 mgs.)	0.30	0.44	0.20*	0.18	0.07	0.09	0.13*	0.12	0.32*	0.10
Male Rats	56	Control	2.51	1.39	3.24*	1.08	0.18	0.10	1.16*	0.39	1.23*	0.24
		Stilbestrol (30 mgs.)	0.19	0.16	0.16*	0.12	0.09	0.08	0.13*	0.10	0.14*	0.09
		PMS + Stilbestrol	0.73	0.62	2.66*	1.61	0.17	0.15	1.78*	1.08	1.31*	0.72

* Differences significant at 1% level.

The experiments involving chickens also consisted of two phases. The first was planned to obtain information on the effect of prolonged action of stilbestrol on the physiology and the organ weights of males. In a second experiment the response of estrogenized cocks to FSH and LH was studied. The first experiment was set up in the same manner as was outlined for the rat trials, the last group of birds being killed 35 days after the implants were made. Three normal controls and 4 estrogenized chickens were killed at the end of each of the 7 day intervals.

RESULTS

Rats: The results obtained following stilbestrol inhibition are in agreement with the traditional expectations of the effects of estrogen on the pituitary gland.

Statistically significant (at the 1% level) reductions were obtained in body weights, testes and seminal vesicle weights (Table 1). The differences between the normal controls and the stilbestrol treated males with regard to adrenal, thyroid, and pituitary weights were statistically not significant, but in all cases these glands were heavier (in percent of body weight) in the inhibited males than they were in the controls.

The testes of estrogenized rats were reduced to about $\frac{1}{3}$ of the weight of the testes of the controls during the shorter experimental period and to about 1/10 of normal during the longer inhibition period. In proportion to the body weight, the seminal vesicles were only slightly smaller in the experimental group during the shorter inhibition period. This difference, however, was much more pronounced after the more prolonged inhibition. On an actual-weight basis, the seminal vesicles did not stop increasing in weight in either group in spite of the complete arrest of testes growth. This was explained by the finding on histological study of these structures that large amounts of connective tissue were laid down presumably as the result of prolonged estrogen treatment. The epithelium of the seminal vesicles was, however, of the typical castrate appearance and the glandular portion of this structure was found by microscopic examination to be completely atrophic. Results of injection of PMS into stilbestrol inhibited males showed that, in spite of prolonged inhibition of the pituitary with estrogen both the testes and the seminal vesicles retained their sensitivity to their respective trophic hormones (Fig. 1).

The males injected with PMS did not show any difference in the rate of gain in body weight from their untreated controls but they continued to gain after the body weights of controls reached a plateau. The final body weight of the inhibited males treated with PMS was significantly higher than that of the controls. It is possible that this growth response is due, at least in part, to a thyrotrophic effect of PMS. In addition to this experiment, thyrotrophic action of PMS

was also noted in tests which involved baby chicks. Whether this effect is due to thyrotrophic action of PMS itself or whether it is due to the release of thyrotrophic hormone from the animal's own pituitary under the influence of PMS, remains problematic. It appears possible that the growth stimulating effect of PMS noted here and elsewhere may be meditated through the thyroid.

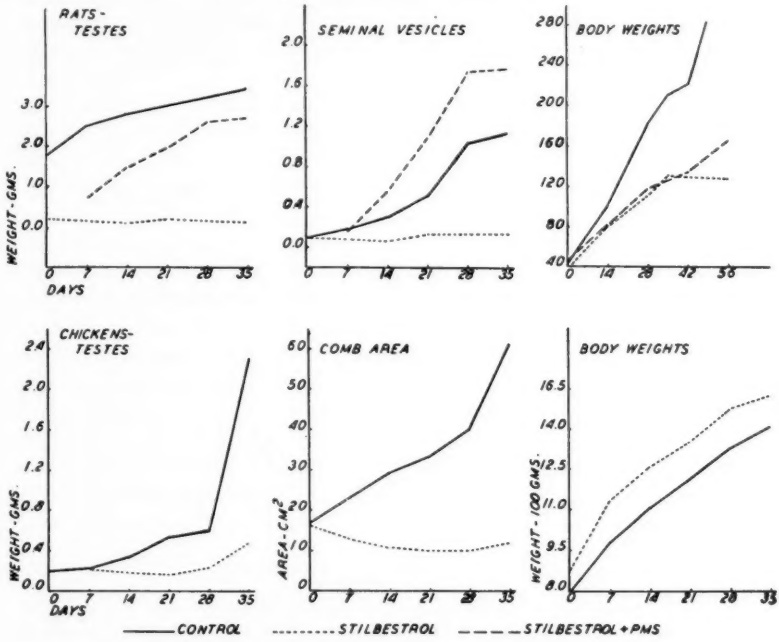

FIG. 1. Comparison of the effects of stilbestrol inhibition on body weights, comb areas, testes weights, and seminal vesicle weights of chickens and rats.

Chickens: In general, the same effects that were observed in the rat also held true for the chicken. The major difference between the responses of the two species lies in the fact that the dosage of stilbestrol used (15 mg. per animal) was not enough to cause any change in the rate of gain in the chicken. At the level given, stilbestrol led to unusual deposition of abdominal and subcutaneous fat, but the rates of gain for the treated group and their controls were not significantly different. The differences between the testes weights and the comb areas between the treated and untreated cocks were very great and statistically significant at the 1% level (Table 1). The differences in weight between the other organs measured were due to chance alone.

Stilbestrol inhibition reduced testes weights within two weeks after

the implant to 0.15% of body weight, while in the controls the testes weight was 0.31%. By the fifth week after the implant the inhibited testes were 0.03% of body weight while the controls had testes which comprised 1.64% of body weight. For the whole experimental period the average testes weights of the inhibited birds were 0.13% while those of the controls were 0.56% of body weight. The reduction in testes weight during the first 4 weeks of stilbestrol inhibition is not as great as that observed following hypophysectomy but is as pronounced at the fifth week as has been observed after surgical hypophysectomy.

Unlike the testes, the comb reaches its minimum size and levels off within 7 to 14 days after silbestrol implantation (and incidentally also after hypophysectomy). In younger males and in those in which the comb is small, complete regression occurs within a minimum of 7 days, while this process requires a slightly longer time in older birds and in those in which the comb is developed beyond average. The rapidity of comb regression indicates that in both surgical and physiological removal of the pituitary, circulating gonadotrophic hormones, and hence androgen, reach a minimum level within a very short time. Evidence of nonsupport of the comb by androgen is apparent almost immediately (1 to 2 days) after either hypophysectomy or stilbestrol implantation. One of the main advantages of using physiologically or surgically hypophysectomized cocks lies in the fact that either incomplete inhibition of the pituitary or partial hypophysectomy become immediately obvious because of non-regression or continued growth of the comb.

EFFECTS OF GONADOTROPHIC HORMONES ON COCKS

In a previous paper Nalbandov, Meyer, and McShan (1946) have shown that hypophysectomized cocks are sensitive test animals for even traces of luteinizing hormone. It was shown that while preparations of FSH free from LH do not cause comb growth, they are capable of causing marked increases in testes size. Histological study of the testes confirmed the expectation that LH causes an increase in testes size by increasing the amount of interstitial tissue, while FSH has the same effect by its action on the proportion and size of the seminiferous tubules. Preparations of FSH contaminated with small amounts of LH were found to cause a significant increase in testes weight but no significant increase in comb area. Invariably, however, such a preparation did cause a reddening of the head region which is characterized in the untreated hypophysectomized bird by a grey pallor. Flushing of the skin of the head region, reddening and eventual growth of the comb are the result of the action on the testes of LH. The intensity and the degree of the response are directly correlated with the amount of LH injected or the ratio of this hormone to FSH if it is injected in a mixture with FSH.

Responses identical to those described for the surgically hypophysectomized cock were found in males inhibited with stilbestrol (Table 2). FSH alone can be distinguished from all preparations containing LH by the fact that the former never produces increases in testes weight of more than 60% over the controls while all LH preparations (at the levels injected) invariably caused testes weight increases of at least 100%. Pure FSH, FSH contaminated with LH, unfractionated anterior pituitaries, and pure LH produced the same effects on estrogen-inhibited males as they did on hypophysectomized males.

TABLE 2. COMPARISON OF THE EFFECTS OF DIFFERENT GONADOTROPES ON THE ESTROGENIZED COCK

Hormone injected (after stilbestrol inhibition)	Color of head region and comb	Size of comb	Average testes weight (gms.)	Tubule diameter
None	pale grey	+	.28	+
FSH (pure)	pale grey	+	.39	++++
FSH and "trace" of LH	red	+ to +++	.60	++++
Whole sheep pituitary	red	++++	.64	++++
LH (pure ?)	red	++++	1.00	+ (or ++ ?)
PMS	red	++++	.67	++++
Untreated	red	normal	9.45	normal

In both hypophysectomized and estrogenized cocks maximum comb size following treatment with LH-containing gonadotrophic hormones was reached within 9 to 15 days after the first injection. For reasons which are to be discussed in a subsequent paper the comb size, and to some extent the testicle size, then decreased in spite of continued injection. Within about 10 days after reaching a maximum area, the comb returned to its preinjection size and became completely atrophic, while the testes leveled off or were reduced in size somewhat. Estrogenized or hypophysectomized males which have been treated once with an LH containing gonadotrophic hormone never again responded to such treatment, precluding the possibility of using such males more than once. This peculiarity is restricted as far as known to the chicken (possibly birds in general), and may not hold true for the rat although the evidence on this point is not complete.

THE USE OF COCKS AS TEST ANIMALS FOR GONADOTROPHIC HORMONES

The results discussed in the previous section make it possible to make the following recommendations for the use of males inhibited with stilbestrol as test animals for gonadotrophic hormones. The male rat appears to be as sensitive as is the chicken. The chicken has the advantage over the rat of having an externally visible and measurable secondary sex character, the comb, which is very sensitive to

even small doses of LH and hence androgen. It is also much cheaper than the rat and in many instances more easily available.

White Leghorn males were used because of their great sensitivity to hormones, their small size and the fact that they are cheap, occasionally being given away by hatcheries. They can be used at any age from 30 to 200 days of age. A 15 mg. pellet of stilbestrol is implanted subcutaneously and at the same time a measurement of the height and length of the comb is made. Further comb measurements are made on days 7, 9, 11, and 13 after the treatment. With younger birds the comb usually reaches its smallest size on day 7, and as soon as a plateau is reached injections are begun. Satisfactory tests are obtained with either 8 injections in 4 days or 8 or 16 injections in 8 days. A final comb measurement is obtained after the last injection, the chickens are killed and the testes removed for weighing. The stilbestrol pellet can be recovered and used over again although it is suggested that at least two "used" pellets be implanted to assure adequate inhibition. As already mentioned, estrogen inhibited or hypophysectomized birds once completely stimulated with an LH-containing hormone preparation lose their ability to respond to such mixtures and for that reason can not be used for the testes more than once.

DISCUSSION

While estrogenized male rats were found useful as test animals for gonadotrophic hormones, estrogenized cocks are preferred for this purpose in this laboratory. In both the rat and the cock sufficiently complete physiological hypophysectomy can be produced with stilbestrol to permit them to be used as test animals for gonadotrophic hormones. Estrogenized cocks appear better suited for qualitative and quantitative gonadotrophic hormone assays because they possess the externally visible and measurable secondary sex character, the comb, which was found to be a reliable and sensitive indicator for endogenous androgen. Furthermore the coloration of the head region was found to be a sensitive indicator for traces of luteotrophic hormone, such traces causing a reddening of the head skin. The obvious advantage of estrogenized over hypophysectomized animals lies in the ease of their preparation.

Estrogen inhibition has been tried on chickens of many age ranges and chicks as young as 30 days were found as useful as were older birds. In general, animals less than 6 months of age were found more sensitive to test hormones and easier to inhibit with stilbestrol than older birds. Injected gonadotrophic hormones cause more uniform increases in the testes of younger birds than they do in those of older inhibited males.

The estrogenized chicken is peculiarly well suited as a test animal for the qualitative and quantitative characterization of gonadotrophic hormones. Its use makes it easy to distinguish between pure FSH and

preparations of FSH contaminated with even small amounts of LH. The test is based on the "all or none" response of the comb and, provided the inhibition of the pituitary is complete, is a more reliable indicator of LH than are the ovaries of the immature hypophysectomized or intact rat. By taking into consideration comb size, testes size and the histological appearance of the testes, it is possible to determine whether an LH preparation is contaminated with small or large amounts of FSH. Although the data on this point are not sufficient, indications are that if a preparation of LH is completely free from FSH, it will not cause any increase in tubule diameter over that found in uninjected controls.

Because of the nature of the test and its reliability, the numbers of animals necessary for the characterization of a gonadotrophic preparation are smaller than would be required if female rats were used.

SUMMARY

In immature male rats and chickens the subcutaneous implantation of 15 mg. of diethylstilbestrol causes a complete inhibition of the animal's own pituitary. This in turn leads to a complete regression of the testes and atrophy of the secondary sex organs supported by androgen.

This effect makes it possible to use such estrogenized males as test animals for the characterization of gonadotrophic hormones. This substitute for surgical hypophysectomy has many obvious advantages over the more time consuming technique.

Estrogenized immature cocks were found to be particularly well adapted to serve as test animals for quantitative and qualitative assay of gonadotrophic preparations. By considering the changes produced in comb size, testes weight, and testes histology it is possible to distinguish positively between pure FSH, FSH and LH mixtures, and pure LH preparations, and naturally occurring mixtures of the two hormones.

REFERENCES

NALBANDOV, A. V., AND L. E. CARD: *J. Exper. Zool.* 94: 387. 1943.
NELBANDOV, A. V., R. K. MEYER AND W. H. MCSHAN: *Endocrinology* 39: 91. 1946.

FORMATION OF SECONDARY DECIDUOMATA IN SPAYED MICE AND LACTATING RATS

P. CONSTANTINIDES

From the Institut de Médecine et de Chirurgie expérimentales,

UNIVERSITÉ DE MONTRÉAL, MONTRÉAL, CANADA

INTRODUCTION

IT HAS BEEN known since the classical observation of Loeb (1907) that the properly sensitized uterine endometrium responds to a traumatic stimulus with a rapid local proliferation that imitates the maternal portion of a placenta.

This growth has been named a deciduoma or placentoma and it is mainly during the last decade that our knowledge about its morphology and experimental physiology has been considerably advanced as a result of the work of Selye and McKeown (1935), Krehbiel (1937), Rothchild et al. (1940), Astwood (1939), Atkinson (1944) and others.

We now know that a deciduoma develops only under highly specific hormonal conditions, that it inevitably and rapidly involutes 5–6 days after its coming into existence, that its life span cannot be appreciably prolonged beyond that time and that a "metrial gland" (another short-lived structure) develops when the deciduoma starts to regress, only to completely disappear after 5 more days.

However, a number of important questions have remained unsettled. One of these has been the subject of a controversy in recent years: it has been repeatedly affirmed and denied that a (primary) deciduoma, once established, inhibits the development of a subsequent (secondary) deciduoma.

The earliest relevant statement came from Selye and McKeown (1935) who, in the course of other extensive studies on the experimental physiology of deciduomata, observed that in a series of 11 lactating rats, secondary deciduomata developed in only six instances.

Subsequently, Rothchild and Meyer (1942) stated without data that primary deciduomata do not inhibit secondary deciduomata in the spayed rat.

Next, Lyon and Allen (1943), confirmed Selye and McKeown's original observation in lactating rats, and as they were able to overcome the inhibition with estrogens, suggested that the growing primary deciduoma might consume estrogen.

Two years later, Atkinson and Leathem (1945) reported a complete inhibition of secondary by primary deciduomata in the spayed

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mouse but did not speculate on the nature of the mechanism.

It was only recently that the problem was approached with more quantitative methods. Peckham and Greene (1947) studied the inhibition in spayed rats and lactating rats with adjusted litters, applying four stimuli per uterine horn and thus making it possible to assay the intensity of the response. They also measured the size of each deciduoma (maximal diameter of the unfixed tissue), calculated statistical significance of results and checked for the non-specific effect of surgical trauma by performing mock-operations in place of the primary stimulation in control groups. They found no significant depression in either the number or the size of secondary deciduomata in the lactating rat. In the spayed rat they observed a significant depression in the size, not in the number, of secondary deciduomata timed similarly and preceded by a mock-operation instead of a primary stimulation. Hence they concluded that the depression of the size was due to the non-specific effect of surgical trauma and prolonged injections.

In summary then, the pertinent literature suggests that there is no specific inhibition in the spayed rat, that it exists in the spayed mouse, that it is doubtful in the lactating rat and that whatever inhibition is observed is due to non-specific effects.

As the whole question has possible implications in the fields of antitumorigenesis, implantation physiology and stress pathology, it was the purpose of this study to investigate the following points:

1. Does the inhibition exist in the spayed mouse or the lactating rat?
2. Is the time interval between the two stimuli essential for the inhibition, i.e. is the metrial gland involved?
3. Do non-specific factors depress the deciduoma response?

MATERIALS AND METHODS

Female Wistar albino rats weighing 250–300 gm. were used for the lactation experiment and female Swiss albino mice weighing 25–30 gm. for all other experiments. The mice received a daily subcutaneous injection of 1 mg. progesterone in 0.05 ml. maize oil for varying periods of time after spaying. The uterine trauma consisted in passing a #50 black cotton thread transversely through the uterus and tying it loosely. In almost all instances three threadings were applied per horn. Experimental and control groups were run simultaneously. The deciduomata were assayed 48 hours after fixation of the uteri in 10% formalin. The evaluation of the response was based on three criteria:

1. *The number of deciduomata developing per horn.* We preferred to express it as mean response per horn instead of pooling all positive responses in a group and expressing them as a percentage of the total possible number of responses. In this way we were able to calculate standard errors of the means and avoided the erroneous comparison of percentages between unequal groups.

It should be noted that the counting of macroscopically unquestionable deciduomata alone frequently misses small but microscopically definite reactions. It sometimes happened that after histologic examination the number of positive responses was found to be considerably higher than before. Step-serial sections were therefore done in every macroscopically negative or doubtful instance.

2. *The maximal diameter of the deciduoma* measured at an axis perpendicular to the mesometrial-antimesometrial axis. All measurements were made by the same observer to the nearest 1/64 of an inch and expressed in this unit. But the diameter does not always reflect the real size of the deciduoma. An oval or oblong deciduoma may have a greater total volume than a spherical one of the same diameter.

3. *The mass of the deciduoma-bearing horn in milligrams.* This third criterion was introduced in order to get a direct expression of the quantity of the newly proliferated deciduoma tissue. It should be noted that the range of weight of a non-stimulated horn under progesterone is 15–35 mg., with a mean of 22. After stimulation, the horn may weigh from 100 to 350 mg. depending on the stage of the deciduoma development. In our opinion, the weight was the most reliable index of the intensity of the response.

The standard error (ϵ) of any mean was calculated from the formula

$$\sqrt{\frac{\Sigma d^2}{n^2 - n}};$$

the standard error of the difference between two means from the formula

$$\sqrt{\epsilon_1^2 + \epsilon_2^2};$$

the probability that the difference between two means is due to chance (P) from Fischer's table of t. Any difference with a P greater than 0.01 was considered not significant.

RESULTS

(A) *Spayed Mice.* The experiments with spayed mice can be classified in the following five groups:

a) *Heterolateral application of primary and secondary stimulus with variation of the time interval between the two stimuli.*

These experiments were done in order to find out whether the reported desensitization of the contralateral horn by a primary deciduoma was a function of the time that separated the two stimulations. Assuming that a specific inhibition existed, there were reasons to expect that at a certain time it would be maximal, while later on it would disappear.

For this purpose various groups of mice were spayed and beginning 48 hours later injected with 1 mg. progesterone daily until they were killed. On the 4th day after commencement of the progesterone treatment the left horn was stimulated in all experimental groups, while a laparotomy was simultaneously performed in all controls. The right horn was stimulated in both experimental and control

groups at different intervals (3, 6, 8 and 11 days) after stimulation of the left horn, a pair of experimental and control groups being dedicated to each interval. All animals were killed four days after stimulation of the right horn. The characteristics of the right horn deciduomata as well as the mean number of the left horn responses are contained in Table I. (The experiment with an interval of 3 days between the two stimulations was performed twice.)

TABLE I

Time interval between the two stimulations	Group	Number of animals	Mean number of decid. per right horn ± ε	Mean diameter of right horn decid. ± ε (in 1/64″)	Mean weight of right horn decid. ± ε (in mg.)	Mean number of decid. per left horn ± ε
3 days. First perform.	Exp.	10	2.6±0.3	6.5±0.3	110±12	2.8±0.1
	Control	7	3.0±0	6.3±0.1	97±4	
	P		0.2	0.6	0.2	
3 days. Repetition	Exp.	13	2.8±0.15	6.7±0.8	118±13	2.7±0.2
	Control	13	2.7±0.2	6.1±0.6	105±11	
	P		0.6	0.6–0.5	0.5	
6 days.	Exp.	8	2.5±0.3	6.3±0.4	103±12	2.8±0.2
	Control	10	1.9±0.4	5.2±0.5	69±9	
	P		0.3–0.2	0.1	0.05	
8 days.	Exp.	9	2.1±0.4	6.4±0.7	119±31	2.7±0.1
	Control	6	2.6±0.4	6.5±0.6	114±49	
	P		0.6	0.9	0.9	
11 days.	Exp.	7	2±0.3	4.8±0.3	58±7	2.6±0.2
	Control	7	1.9±0.3	4.9±0.6	64±11	
	P		0.2	0.9	0.7	

The results clearly indicate that there is no significant difference between the number, size or mass of the deciduomata that were preceded by other deciduomata and those that were preceded by a laparotomy, whether the two stimulations were 3, 6, 8 or 11 days apart. One is driven to the same conclusion if the mean values of all experimental and all control groups are pooled as in Table II.

It is striking that the deciduomata that developed after 16 days of progesterone treatment (the 11 days interval between the two stimuli)—whether preceded or not by other deciduomata—were significantly smaller in size and mass than those of every other single group except the control group in the 6 days interval. Due to its

TABLE II

Group	Number of animals per group	Mean number of decid. per right horn ± ε	Mean diameter of right horn decid. ± ε (in 1/64″)	Mean weight of right horn decid. ± ε (in mg.)	Mean number of decid. per left horn ± ε
Experimental	47	2.4 ± 0.14	6.1 ± 0.42	101 ± 11	2.7 ± 0.1
Control	43	2.4 ± 0.25	5.8 ± 0.3	90 ± 9	
P		0	0.6	0.5	

singular occurrence, the latter irregularity must be attributed to chance.

b) *Effect of extirpation of the primary-deciduoma-bearing horn on secondary deciduomata.*

One would expect that if a primary deciduoma exerts any influence on a secondary, its extirpation prior to the second stimulation should remove that influence. For that purpose eight mice were spayed, and beginning 48 hours later injected daily with 1 mg. progesterone until they were killed. On the 4th day after commencement of the injections the left horn was stimulated. On the 8th day, this horn with its fully developed deciduomata was extirpated and on the 11th day, the right horn was stimulated. The animals were killed on the 15th day.

A control group was run simultaneously, timed similarly and subjected to the same experimental procedure, except for the fact that the extirpation of the left horn deciduomata was omitted.

The right horn deciduomata of both groups are compared in Table III.

TABLE III

Group	Number of animals per group	Mean number of decid. per right horn ± ε	Mean diameter of right horn decid. ± ε (in 1/64″)	Mean weight of right horn decid. ± ε (in mg.)	Mean number of decid. per left horn ± ε
Hysterectomized	8	2.1 ± 0.4	5.2 ± 0.4	102 ± 20	3 ± 0
Intact	8	2.5 ± 0.3	6.3 ± 0.4	103 ± 12	2.8 ± 0.2
P		0.4	0.1–0.05	0.9	

It can be seen that the removal of the primary deciduomata did not affect the secondary deciduomata.

That the insignificant depression of the average values for number and size of secondary deciduomata in the hysterectomized group was not due to the greater surgical trauma in that group was further proven in subsequent experiments designed to study the effect of non-specific stress on deciduomata.

c) *Homolateral application of primary and secondary stimulation.*

If a deciduoma inhibits another deciduoma by either consuming or producing a hormone, the resulting gradient of inhibition should have its maximum in the uterine area immediately adjacent to the primary deciduoma. To test this, six mice were castrated and beginning 48 hours later, received daily injections of 1 mg. progesterone until they were killed. On the 4th day of progesterone treatment the left horn was stimulated at two points separated by a distance of about one cm. On the 11th day, a single stimulus was applied between the two deciduomata that had developed in the left horn as a result of the stimulation on the 4th day. The right horn was also stimulated singly on that occasion. Three days later the animals were killed. The secondary deciduomata of the left and right horn are compared with each other in Table IV.

TABLE IV

Group	Number of animals	Total number of secondary decid.	Expected number of secondary decid.	Mean diameter of secondary decid. ± ϵ (in 1/64")	Total number of primary decid.	Expected number of primary decid.
Secondary decid. of the left horn (=homolateral to primary decid.)	6	6	6	6±0.3	11	12
Secondary decid. of the right horn (=contralateral to primary decid.)	6	6	6	6±0.3		

It is evident from the data that there is no difference between the number or size of secondary deciduomata developing in the horn containing the primary deciduoma and those of the contralateral horn.

d) *Effect of non-specific stress on deciduomata.*

Peckham and Greene (1947) concluded from some of their experiments that the non-specific effect of surgical trauma and prolonged treatment was responsible for the significant depression of the secondary response in spayed rats and also suggested that the same non-specific factors "might have a greater influence on the (deciduoma) reaction in the mouse, i.e., instead of merely decreasing the size of the secondary deciduomata . . . they might prevent their formation."

The following experiment was designed to test this hypothesis: Four groups of spayed mice were treated with progesterone in the usual way, traumatized in the right horn on the 4th day and killed on the 8th day of treatment. Of these four groups one served as non-stressed control, while each of the other three was exposed to a different type of general stress during the entire period of progesterone treatment.

Cold, formalin injections and extensive operative trauma with repeated narcoses were the types of stress employed.

In the group exposed to cold the animals were put in the refriger-

ator at 0°C twice daily for 5 hours with an hour's rest period between the two expositions.

In the formalin treated group the animals were injected twice daily with 0.05 ml. of a 10% formalin solution subcutaneously.

The third experimental group was unilaterally nephrectomized, splenectomized and partially pancreatectomized on the second day of progesterone treatment and subjected to deep ether anesthesia twice daily for ten minutes during all subsequent days.

The characteristics of the deciduomata that developed in those four groups are compared with each other in Table V.

TABLE V

Group	Number of animals	Mean number of decid. per horn $\pm\epsilon$	Mean diameter of decid. per horn $\pm\epsilon$ (in 1/64″)	Mean weight of decid. per horn $\pm\epsilon$ (in mg.)
I. Non-stressed control	13	2.7 ± 0.2	6.1 ± 0.6	105 ± 11
II. Operations+anesthesia	6	3 ± 0	7 ± 0.5	122 ± 19
III. Cold	8	3 ± 0	7.4 ± 0.35	119 ± 18
IV. Formalin*	4	2.5 ± 0.5	6.3 ± 1.2	99 ± 27
P of I versus II		0.2–0.1	0.3	0.5
P of I versus III		0.2–0.1	0.2–0.1	- 0.5

* The P of I versus IV was not calculated as only 4 animals survived in the formalin group (from an original number of 12). It seems certain however that formalin did not depress the deciduoma formation any more than the two other types of stress.

It is obvious that considerable non-specific stress applied continuously before, during and after the uterine stimulation does not depress the deciduoma formation in the spayed mouse. The higher average values for number, size and mass of the deciduomata in stress groups II and III, although not significant and probably due to the smaller number of animals in those groups, suggested the possibility that stress itself might synergize progesterone in the target tissue either through discharge of a luteoid hormone from the adrenals or through some other mechanism. It would be interesting to see whether stress alone, without progesterone, could sensitize the uterus. For that reason four mice were spayed; starting 48 hours later they were put in a refrigerator at 0°C for ten hours daily and exercised in the revolving chamber for two hours daily until they were killed. On the 4th day after commencement of stress the uterus was stimulated in the standard way and on the 8th day the animals were killed. Another 4 animals served as non-stressed controls; of these, 2 received progesterone and 2 did not.

No deciduomata developed in any of the experimental animals.

Histologic examination of the uteri revealed that by Hooker's criterion (1945) there were no demonstrable luteoid or folliculoid hormone-levels in the organism. The control animals reacted as expected.
(B) *Lactating rats.* In the experiment of Peckham and Greene (1947) with lactating rats the second stimulus was applied 3 days after the first one. As earlier investigators who obtained a depression of the secondary response had applied the second stimulus later, at a time when a "metrial gland" had formed as a result of the first stimulation, it was considered appropriate to separate the two stimuli by an interval of 6 days.

In an experimental group of seven lactating rats the left horn was traumatized on the fifth day and the right horn on the 12th day after parturition. The animals were killed on the 16th day.

In another control group of seven lactating rats, timing and operations were identical except for the fact that a mock-operation was performed in place of the stimulation of the left horn. The litters were adjusted to 7 ± 1 sucklings per lactating animal in both groups, throughout the experiment.

The characteristics of the deciduomata resulting in both groups from stimulation on the 12th day after parturition are tabulated in Table VI.

TABLE VI

Group	Number of animals per group	Mean number of decid. per right horn $\pm \epsilon$	Mean diameter of right horn deciduomata $\pm \epsilon$ (in 1/64")	Mean weight of right horn decid. $\pm \epsilon$ (in mg.)	Mean number of decid. per left horn $\pm \epsilon$
Experimental	7	2.4 ± 0.4	9 ± 1	339 ± 64	2.7 ± 0.2
Control	7	3 ± 0	9.4 ± 0.5	413 ± 59	
P		0.2	0.7	0.5	

The results suggest that there is no significant difference between the number, size or mass of deciduomata that were preceded by other deciduomata and those that followed a mere laparotomy.

CONCLUSIONS

(A) *Spayed Mice.* It seems justified to conclude that the presence in a uterine horn of a deciduoma or a "metrial gland" at any stage of development does not hinder the development of another deciduoma in the same or the contralateral horn.

Furthermore, it appears that the deciduoma response is neither increased nor decreased by acute, non-specific damage to the organism, possibly because the latter fails to cause the discharge of threshold quantities of luteoid or folliculoid hormones from the adrenals. It is remarkable that even a stress so severe as to kill 75% of the

animals (formalin injections) did not influence the deciduoma response in the survivors.

Finally, there was a tendency towards a decrease of the mean number of responses per horn with increasing length of progesterone pretreatment; stimulation after the longest progesterone treatment (15 days), whether preceded or not by other deciduomata, resulted in responses that were markedly and significantly weaker than after almost every shorter treatment (cf. Table I). This suggests that long lasting progesterone levels diminish the deciduoma response. We seem to deal here with another instance of target refractoriness developing after protracted action of a steroid hormone.

(B) *Lactating Rat.* We could confirm Peckham and Greene's conclusions that there is no inhibition of secondary by primary deciduomata in the lactating rat.

The difference between our own conclusions and those of some earlier investigators is probably due to the great individual variation of the deciduoma reaction and to the difficulty of its quantitative assessment in unequal groups, on the basis of macroscopical counts alone, without further criteria or statistical evaluation of data.

SUMMARY

The formation of a deciduoma in one uterine horn of a spayed mouse is not influenced by the pre-existence in either horn of another deciduoma or metrial gland at any stage of development.

Acute non-specific stress does not affect the deciduoma response in the spayed mouse, nor does it cause the secretion of demonstrable luteoid or folliculoid hormones from the adrenals.

Protracted action of progesterone diminishes the deciduoma response in the spayed mouse.

Peckham and Greene's observations in the lactating rat are confirmed.

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REFERENCES

ASTWOOD, E. B.: *J. Endocrinol.* 1: 49. 1939.
ATKINSON, W. B.: *Anat. Rec.* 88: 271. 1944.
ATKINSON, W. B., AND J. H. LEATHEM: *Proc. Soc. Exper. Biol. & Med.* 58: 283. 1945.
HOOKER, C. W.: *Anat. Rec.* 93: 333. 1945.
KREHBIEL, R. H.: *Physiol. Zool.* 10: 212. 1937.
LOEB, L.: *Zentrabl. f. allg. Path. u. path. Anat.* 18: 563. 1907.
LYON, R. A., AND W. M. ALLEN: *Anat. Rec.* 86: 417. 1943.
PECKHAM, B. M., AND R. R. GREENE: *Endocrinology.* 41: 277. 1947.
ROTHCHILD, I., AND R. K. MEYER: *Physiol. Zool.* 25: 216. 1942.
ROTHCHILD, I., R. K. MEYER AND M. A. SPIELMAN: *Am. J. Physiol.* 128: 213. 1940.
SELYE, H., AND T. MCKEOWN: *Proc. Roy. Soc.*, London, s. B. 119: 1. 1935.

PROGESTERONE AND ESTROGEN IN THE EXPERIMENTAL CONTROL OF OVULATION TIME AND OTHER FEATURES OF THE ESTROUS CYCLE IN THE RAT

JOHN W. EVERETT

From the Department of Anatomy, Duke University School of Medicine

DURHAM, NORTH CAROLINA

INTRODUCTION

SEVERAL YEARS ago it was reported that small daily doses of progesterone given to persistent-estrous rats serve to interrupt the equilibrium between hypophysis and ovaries, inducing sequences of vaginal cycles which closely resemble those in normal animals (Everett, 1940). In a significantly high proportion of these cycles ovulation occurs and new corpora lutea are formed. This finding cannot be explained on the assumption which has been generally held for many years that the principal effect of progesterone upon the hypophysis is suppression of LH secretion. Subsequent studies have made it more and more apparent that under certain conditions progesterone facilitates the release of luteinizer.

In 1943 we reported that if persistent estrus is interrupted experimentally by a single injection of progesterone, a diestrous interval of 2 to 3 days usually occurs followed by the typical proestrous smear picture and then full cornification which continues if no further treatment is given. Newly grown follicles persist under these conditions. If, however, a second injection of progesterone is given during late diestrus or early estrus, luteinization is usually induced and the cycle is completed. Sequences of such cycles can thus be maintained by an injection of 0.5 to 1.0 mg. of progesterone during proestrus of each cycle. More recently we have extended the investigation to normal rats. In a preliminary account (Everett, 1944) it was reported that in normal rats which exhibit predictably regular 5-day cycles the injection of 1.0 mg. of progesterone about noon on the third day of diestrus serves to advance ovulation time approximately 24 hours. Ovulation occurs during the night following injection and, of equal significance, the vaginal smears are usually cornified the next morning. On the other hand, no advance of ovulation time nor hastening of vaginal changes is obtained in 4-day cyclic rats injected 24 hours before the expected proestrus. This particular observation makes it especially evident that other factors intrinsic in the cycle are of primary impor-

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tance and that the action of progesterone is secondary to these. In this connection it should be recalled that, during diestrous intervals experimentally induced in persistent-estrous rats, a single injection of progesterone early in diestrus is ineffectual in causing completion of the ensuing cycle (Everett, 1943), while it is most effectual if given near the onset of heat when estrogen levels are undoubtedly rising or already maximal.

Since the initial experiments of Hohlweg (1934) it has been repeatedly indicated that estrogen itself causes liberation of luteinizing hormone[1] from the rat hypophysis (Lane, 1935; Fevold, Hisaw and Greep, 1936; Hohlweg and Chamorro, 1937; Westman and Jacobsohn, 1938). It was also recently demonstrated in the pregnant or pseudopregnant rat that injection of a few micrograms of estradiol benzoate causes ovulation about 36–40 hours afterward (Everett, 1947). The presence of active corpora lutea thus appears quite compatible with LH liberation under some circumstances.[2] Yet under other circumstances progesterone seems to prevent LH release. Selye, Browne and Collip (1936) reported that daily injection of 4 mg. of progesterone in rats suppresses vaginal cycles. Phillips (1937) later demonstrated that daily injection of as little as 1.5 mg. will postpone indefinitely the next expected estrus if the treatment is begun during early diestrus. Dempsey (1937) noted in the guinea pig that daily injection of progesterone specifically prevents the advanced stages of follicle maturation. This was regarded as evidence for suppression of LH secretion. Using the augmentation reaction in rats treated daily with progesterone, Astwood and Fevold (1939) reached a similar conclusion. Makepeace, Weinstein and Friedman (1937) had found in the rabbit, meanwhile, that injection of progesterone for 5 days prevents ovulation after mating on the sixth day. Subsequently, Friedman (1941) reported that treatment of estrous rabbits for 24 to 72 hours greatly reduces the number ovulating in response to copper acetate injection.

The experimental results recorded below will, it is hoped, partially resolve the apparent disagreement between these results and our evidence that progesterone facilitates LH release. Preliminary accounts of certain of the observations reported here have been presented elsewhere (Everett, 1944, 1946).

MATERIALS AND METHODS

In the design of the several experiments described below a high degree of predictability was essential, with respect to cycle length, ovulation time and

[1] Or total gonadotrophin (FSH+LH). The expression LH is freely used in this paper in the conventional sense (cf. Hisaw, 1947), with full realization that FSH also enters into preovulatory stimulation of follicles. The phenomena with which we are dealing here may largely express quantitative rather than qualitative differences in gonadotrophin secretion.

[2] A more crucial demonstration of this point appears later in this paper.

other chronologic features of the estrous cycle. To this end, lighting is automatically controlled by a time switch which gives the colony 14 hours of light and 10 hours of darkness daily throughout the year. Room temperature is not controlled, but it has been our experience that only sudden weather changes produce large-scale changes in the cycles. We have been able to recognize such general effects in the colony as a whole by virtue of the fact that vaginal smears are taken daily in a group of 50 to 75 rats, only a small number of which are being treated experimentally at any one time. The remainder thus serve as controls for the experimental group.

We have consistently worked with inbred strains: the "normal" Vanderbilt (Osborne-Mendel) strain, and, for specific purposes, the defective DA strain. Long experience with the former has enabled us, after following the vaginal smear sequences for 2 to 4 weeks, to predict with rare failure the night during which ovulation will occur in a given animal having a consistent history of 4-day or of 5-day cycles. Two substrains of the Vanderbilt stock have been developed. In one of these (designated Va) most of the females have regular 4-day cycles after attaining full maturity. Frequently the first few cycles are 5 days long, but these usually give way eventually to 4-day cycles. From our records we have calculated that if a Va animal has experienced two 4-day cycles in sequence the probability is about 93% that the next cycle will also be 4 days in length. In the second Vanderbilt substrain (designated Vc) nearly all females have regular 5-day cycles. In this group, after two successive 5-day cycles have been observed in a given rat, the probability is 90% that the next cycle will also be 5 days long. There is only a 4% chance that this next cycle will be spontaneously shortened to 4 days and even this small chance may be nearly eliminated by selection of animals showing 3 days of clearly diestrous smears in the third cycle. In the defective DA strain, young virgin females are usually regularly cyclic during the third and fourth months of age, these cycles being mostly of 5 days' duration. Subsequently such animals become persistent-estrous (Everett, 1939, 1942). This condition is well established in nearly all cases before the age of 200 days. In certain instances specified below, first and second generation hybrids between the Vanderbilt and DA strains were employed. These were vigorous animals and most of them proved to have either 4-day or 5-day cycles which were highly predictable.

The vaginal smears are obtained daily at 8 to 10 A.M. by saline lavage, fixed in alcohol after drying and stained with 1% alkaline toluidine blue. The typical daily smear sequence thus found in 4-day cyclic Vanderbilt rats is as follows: *day 1*—(diestrus) heavily leucocytic with occasional remnants of clumps of cornified cells; *day 2*—(diestrus) moderately leucocytic; *day 3*—(early proestrus) absence of leucocytes, numerous small, nucleated epithelial cells among thin, non-nucleated squames (some animals tend to show a few leucocytes among the epithelial cells at this time, becoming clearly proestrous later in the day); *day 4*—(estrus) full cornification. Ovulation occurs during the night preceding day 4. The smear sequences in 5-day cyclic Vanderbilt rats are of two general types: In the first type (A) leucocytic smears are obtained on 3 successive days. On day 4 (proestrus) the smears are free of leucocytes and rich in small nucleated epithelial cells, usually in clumps. On day 5 (estrus) they are fully cornified, occasionally with beginning admixture of leucocytes and nucleated squamous epithelial cells. In the second type (B)

the smear on day 3 is free of leucocytes or nearly so and closely resembles the proestrous smear seen on day 3 of the 4-day cycle. On the next morning the smear is either of the late proestrous type, containing a mixture of small non-nucleated squames and round cells with indistinct nuclei, or slightly more advanced, containing a uniform spread of small cornified cells. On day 5 full cornification is seen as in the first type. Almost needless to say, intergrades between types A and B occur. In either type, ovulation occurs during the night preceding day 5. In the DA strain both types of 5-day cycle are encountered, but more commonly type A.

In predicting the night of ovulation we by no means employ as principal criterion the vaginal smear picture on the preceding day. Rather, the full sequence of smears over several cycles is considered and the events in the current cycle are predicted against this background, using the current smears to indicate any significant irregularities.

Actual ovulation time has been estimated in 4-day cyclic Vanderbilt rats by killing 28 of them between 12:45 and 4:00 A.M. during the night following proestrus. Preovulatory swelling or ruptured follicles were observed in all cases. Ovulation in progress was found as early as 1:10 and as late as 1:55. Completed ovulation (8 or more tubal ova) was found as early as 1:45 and in every case examined after 2:30. Although no such direct information has been obtained in 5-day cyclic rats of either strain, the histological appearance of new corpora lutea obtained from them during the late morning or early afternoon of day 5 is similar to that seen at such times on day 4 of the 4-day cycle.

In our routine procedure, direct visualization of tubal ova is accomplished after killing the animals with illuminating gas, by excising the ampullae and mounting them in physiological saline under a cover slip (Everett, 1947). In the rat, as in the mouse (Burdick and Whitney, 1941), the thin walls of the dilated ampullae allow ready identification of granulosa cell masses and ova during the first day after ovulation. The excised ovaries are examined in saline under dissecting lenses for the tentative identification of newly ruptured follicles.

In most instances described here the ovaries were fixed in Zenker's fluid, embedded in paraffin and serially sectioned at 8 to 10 μ. The routine stain was Harris' hematoxylin followed by a modification of Mallory's tri-acid stain (Everett, 1943).

In the experiments which follow, progesterone was administered subcutaneously in a sesame oil solution containing 5 mg. per ml. Estradiol benzoate was also given in sesame oil by subcutaneous injection. This solution contained 330 μg. per ml. In certain instances small crystals of estradiol weighing approximately 100 to 200 μg. were introduced subcutaneously by means of a 16 gauge hypodermic needle and stylus (Everett, 1947).

RESULTS

Five-day cycle

Advancement of ovulation time by progesterone. In a preliminary report (Everett, 1944) as mentioned above, it was shown that in the 5-day cyclic rat, if progesterone is injected near midday on the third day of diestrus, ovulation time is advanced about 24 hours. This basic

experiment will merely be summarized here (fig. 1, B). The reader is referred to the article cited for additional information. In choosing 5-day cyclic animals for this treatment only those showing distinctly leucocytic smears on the day of injection were employed, to insure against any spontaneous shift to a 4-day cycle. When given as a single injection, timed as indicated above, progesterone causes ovulation to occur approximately 24 hours earlier than it would have occurred without treatment. In ovaries removed about noon on the day following injection of the new corpora lutea appear identical histologically with those which one would find a day later in the nor-

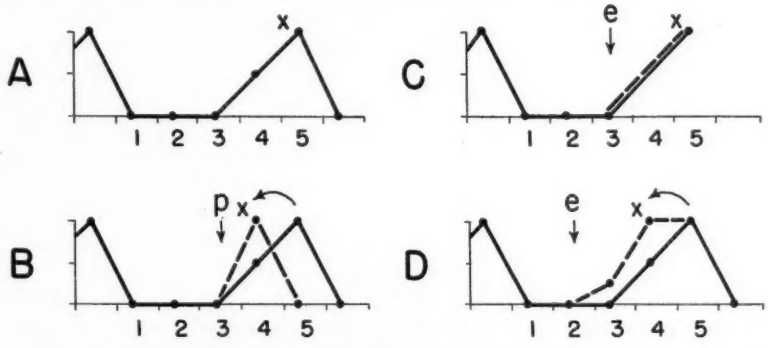


FIG. 1. The 5-day cycle and its experimental modifications. Two units of the ordinate represent a full estrous smear. Time in days is indicated on the abscissa, each unit representing 24 hours (midnight to midnight). Days of the cycle are numbered progressively beginning on the first day of diestrus. Ovulation time is represented by "X".

A. The standard 5-day cycle, ovulation occurring shortly after midnight on day 5.

B. 1.0 to 2.0 mg. of progesterone (p) injected on day 3 accelerates ovulation and vaginal cornification about 24 hours.

C. 33 to 100 μg. of estradiol benzoate (e) injected on day 3 does not modify the cycle during the next 48 hours.

D. 50 μg. estradiol benzoate (e) or implantation of small crystals of estradiol on the second day of diestrus accelerates ovulation and vaginal changes 24 hours.

mal 5-day cycle. Dose levels of either 1.0 or 2.0 mg. of progesterone appear to be equally effective in Vanderbilt rats and in hybrids,[3] a fact which is especially significant when it is recalled that 1.5 mg. daily will suppress ovulation if treatment is begun sufficiently early in the cycle (Phillips, 1937).

The vaginal smear on the morning after induced ovulation is characteristically fully cornified, a fact indicated in figure 1, B, by the peak in the broken line. Although not of maximal size the cells appear in clumps and present a picture quite commonly seen in the 4-day cyclic rat on the morning after spontaneous ovulation.

[3] The failures reported with the larger dose in DA rats (Everett, 1944) may or may not be significant. In the absence of further information in that strain, only the data from Vanderbilt rats and hybrids is considered acceptable at present.

In addition to cases cited in the preliminary report, 5 more positive responses (in Vanderbilt rats) have been obtained. Hence, in 18 of 19 cases (11 Vanderbilt rats, 8 hybrids), ovulation time was advanced about 24 hours and the process of vaginal cornification was significantly accelerated in 17 of the 18 rats. Six controls (2 Vanderbilt rats, 4 hybrids) receiving 0.4 ml. sesame oil in place of the progesterone solution were proestrous on the following day as expected.

Advancement of ovulation by estrogen. There is general agreement that in the rat the full vaginal response to injected estrogen is delayed, so that in primed castrates cornification is not obtained until about 36 to 48 hours after injection (cf. Gustavson, 1939; Jones and Astwood, 1942). With respect to induction of ovulation by estrogen in rats, most information pertains only to immature females where the response is delayed about 4 or 5 days (Hohlweg, 1934; Westman and Jacobsohn, 1938). In the adult female, however, administration of estrogen during early pregnancy is regularly followed by ovulation and new corpus luteum formation within 36–40 hours (Everett, 1947). It is now reported that when estrogen is administered on the second day of diestrus in 5-day cyclic rats, ovulation occurs approximately 24 hours earlier than if no treatment were given (fig. 1, D), again at an interval of approximately 40 hours after injection.

As a preliminary to this particular phase of the study, 4 Vanderbilt rats were injected with estradiol benzoate (33 μg., 3 cases; 100 μg., 1 case) at about noon on the third day of diestrus (fig. 1,-C). During the following two days no modification of the normal vaginal smear sequences was found. Tubal ova were observed at autopsy about 48 hours after injection and the newly forming corpora lutea were exactly comparable histologically with those normally found at that stage of the 5-day cycle. Thus no interference with the ovarian cycle was apparent within that time interval and such doses of estrogen appear entirely compatible with the processes involved in follicle maturation and ovulation.

Subsequently, seven 5-day cyclic Vanderbilt rats were given estrogen during the second day of diestrus (fig. 1, D), between 11:00 A.M. and 3:00 P.M. Five of these received 50 μg. of estradiol benzoate in 0.15 ml. of oil. In the other two cases small crystals of estradiol were introduced subcutaneously. On the following morning the vaginal smears were still somewhat leucocytic except for one case in which an early proestrous smear was obtained. On the second morning all smears were cornified. At autopsy later that day, about 48 hours after estrogen treatment, full sets of tubal ova were found in all cases and the ovaries contained full sets of very new corpora lutea which in histological section appeared closely similar to those found a day later in the normal 5-day cycle. Thus, this treatment accelerated vaginal cornification and advanced ovulation about 24 hours.

Four-day cycle

Non-effect of progesterone injected 24 hours before proestrus. Six 4-day cyclic females (hybrids) were injected with progesterone between 10:30 A.M. and 2:00 P.M. on the second day of diestrus, about one day before proestrus was expected (fig. 2, B). Four of them received 2.0 mg. each and two received 1.0 mg. each. On the following morning the smears were of early proestrous type, no evident modification having been produced by the treatment. At autopsy, about 24 hours after injection, the ovaries presented the typical appearance of proestrus, containing sets of large, clear follicles. Histologically they were identical with those in untreated control rats at the same stage of the cycle. The uteri, however, were slender, in contrast with the distended condition normally seen at this time.

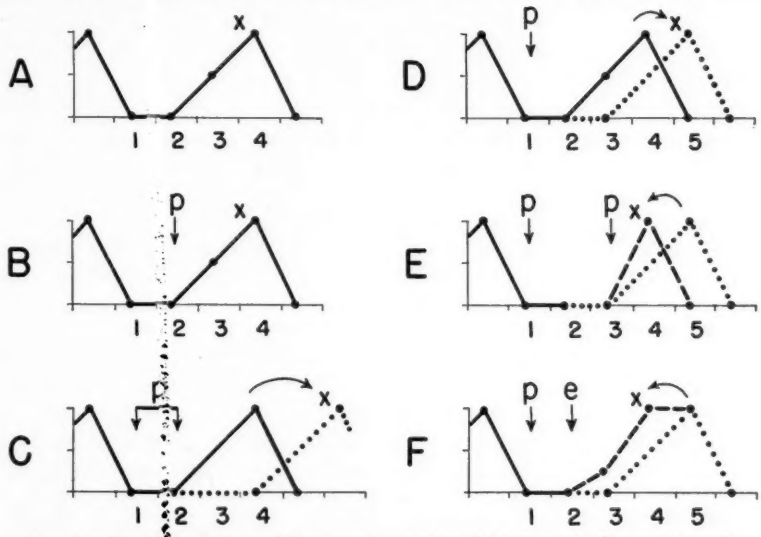


FIG. 2. The 4-day cycle and its experimental modifications. Units on the ordinate and abscissa have the same meaning as in figure 1.

A. The standard 4-day cycle, ovulation occurring 1:00 to 2:30 A.M. early in day 4.

B. Progesterone (p) injected on the second day does not accelerate ovulation and vaginal changes (cf. fig. 1, B).

C. 1.5 mg. of progesterone (p) on days 1 and 2 of diestrus retards the cycle 2 days.

D. 1.5 mg. of progesterone (p) on day 1 of diestrus retards ovulation and vaginal estrus about 24 hours. This is an "artificial" 5-day cycle, now indicated by the numbering below the abscissa.

E. In such an artificial 5-day cycle, additional progesterone on day 3 of diestrus advances ovulation time and vaginal cornification about 24 hours (cf. fig. 1, B and contrast with fig. 2, C).

F. In the artificial 5-day cycle estrogen (e) on the second day of diestrus advances ovulation time 24 hours. (cf. fig. 1, D).

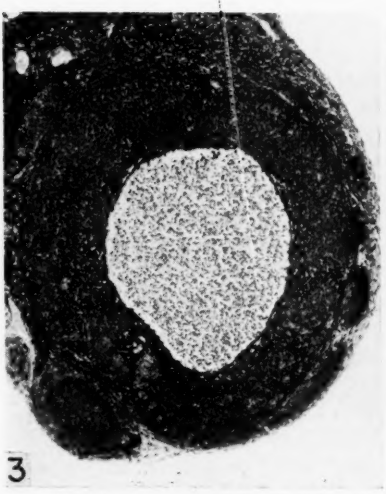
Retardation of the cycle by progesterone injected early in diestrus.

When daily injections of over 1.0 mg. of progesterone are instituted during early diestrus, the diestrus is maintained throughout the period of injection and for 2 days afterward (Phillips, 1937). Then the animal returns to heat. We have repeated this experiment on numerous occasions with the same result. For example, in the particular modification illustrated in figure 2, C, it is seen that, by treating 4-day cyclic females with a limited series of 2 daily injections of progesterone (1.5 mg.) both the onset of vaginal estrous stages and the time of ovulation are retarded about 2 days.

When only one injection of progesterone is given to a 4-day cyclic rat on the first day of diestrus the result is illustrated in figure 2, D: the 4-day cycle is transformed to a 5-day cycle by retarding both the onset of vaginal estrus and ovulation time by about one day. Twenty-nine rats were so treated during a total of 43 cycles. Two different dose-levels were employed. In 23 cycles the amount injected was 1.0 mg. Among 8 such cases in hybrid rats 6 cycles were prolonged one day and 2 were not. In Vanderbilt rats 12 of 15 cycles were prolonged one day. Because of these occasional failures the dose was then increased to 1.5 mg. No failures were encountered among 20 cycles in 18 Vanderbilt rats given this amount. These animals exhibited proestrous smears on the third morning after injection in all cases. Eleven rats were then autopsied without allowing the cycles to continue longer. Their uteri were hyperemic and distended. Their ovaries contained sets of large, clear follicles which were histologically identical with those found during early proestrus in the normal 5-day cycle (fig. 5). They were larger than during proestrus in the 4-day cycle, but gave no evidence of preovulatory swelling. In 10 cases the cycles were continued for at least another day, full cornification appearing at that time. Four were autopsied on that day, on the fourth day post-injection. The reproductive tracts were typical of late estrus. The ampullae of the oviducts were dilated and full sets of tubal ova were demonstrated. New corpora lutea were found, histologically identical with those obtained in normal cycles on the day after ovulation (figs. 3 and 4). In other instances, in which the animals were followed longer, diestrous smears were found on the following morning.

Since these artificially retarded cycles in 4-day cyclic rats are apparently identical with normal 5-day cycles, it appeared desirable to test such cases with respect to the sequellae of injection of additional progesterone on the third day of diestrus or of estrogen on the second day of diestrus. These results are described below.

Effect of a second injection of progesterone during diestrus day 3 of the retarded cycle. Five 4-day cyclic Vanderbilt rats were each given 1.5 mg. of progesterone on the first day of diestrus. Two days later, the third day of diestrus in the retarded cycles, each rat received 1.0 mg. of progesterone between 12:00 and 1:00 P.M. (fig. 2, E). On the following morning the vaginal smears were predominantly cornified in



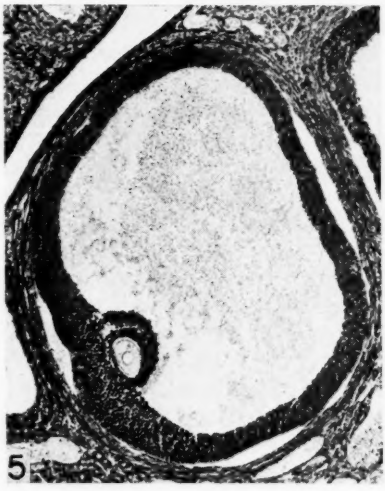


FIG. 3. New corpus luteum at 12:00 Noon on day 5 (estrus) of a standard 5-day cycle (cf. Boling, 1942, fig. 3). 80×.

FIG. 4. New corpus luteum at 12:30 P.M. on day 5 (estrus) of a potential 4-day cycle retarded 1 day by progesterone (cf. fig. 2, D). 80×.

FIG. 5. A follicle typical of day 4 (proestrus) in a 5-day cycle. This specimen was actually obtained during a potential 4-day cycle, retarded 1 day by progesterone injected on day 1 of diestrus (cf. fig. 2, D). Ovaries removed ca. 72 hrs. post-injection. 80×.

FIG. 6. New corpus luteum at 8:30 A.M. on day 4 of an experimental cycle as represented in fig. 2, E. Originally a 4-day cycle; progesterone on day 1 of diestrus and again on day 3 (1:00 P.M.). Closely similar to corpora lutea at this hour of day 4 (estrus) in standard 4-day cycles (6–8 hr. post-ovulatory). 80×.

4 cases, with slight admixture of small nucleated epithelial cells and a few nucleated squamous cells. One smear was recorded as proestrous. The 4 showing cornification were autopsied that morning. Tubal ova and new sets of corpora lutea (fig. 6) were demonstrated in all. The remaining rat was continued until the following morning. At that time the smear had become diestrous and at autopsy the ovaries appeared to contain a set of unruptured follicles and one element tentatively diagnosed as a new corpus luteum. Serial sections of that ovary disclosed 2 large atretic follicles and a corpus luteum, estimated to be about 30 hours old.

Effect of estrogen injected on diestrus day 2 of the retarded cycle. Five 4-day cyclic Vanderbilt rats were injected with 1.5 mg. of progesterone on the first day of diestrus. On the following day (fig. 2, F) each rat received an injection of estradiol benzoate: 50 μg. in 2 cases, 15 μg. in 3 cases. They were autopsied about 48 hours later. The vaginal smears registered little significant effect of the estrogen on the first morning after injection, but were fully cornified on the second morning. Examination of the reproductive tracts disclosed hyperemic, distended uteri. The ampullae of the oviducts were dilated and tubal ova were demonstrated in each case. Newly forming corpora lutea were recognized in all 5 pairs of ovaries.

It is thus apparent that in the artificial 5-day cycle, as in the natural 5-day variety, ovulation time can be advanced by either progesterone or estrogen when the injections are suitably timed.

Attempts to ovulate persistent-estrous rats by estrogen therapy

Non-effect of estrogen alone. In a variety of experiments with persistent estrous rats of the DA strain, single injections or multiple daily injections of estradiol benzoate have consistently failed to induce ovulation or luteinization. Only the more significant of these experiments will be described here. Having failed to produce these effects by estrogen therapy while persistent estrus was in progress and having meanwhile devised a means of inducing cycles in such animals by progesterone treatment (Everett, 1940, 1943), the next logical step was to introduce estrogen during diestrus after one completed "progesterone cycle." For purposes of illustration a sequence of two "progesterone cycles" is shown in figure 7, A. Persistent estrus is interrupted by one injection of 1.0 mg. of progesterone after which a diestrous interval of 2 to 3 days usually follows. If, then, a second injection of progesterone is given during proestrus the cycle is completed. Subsequent cycles are completed if progesterone is given during each proestrus. Ovulation occurs in about 70% of the cases.

Twelve persistent-estrous DA rats were each passed through one progesterone cycle as shown in figure 7, B. Six of these also received 1.5 mg. of progesterone (not shown in the figure) on the first day of the second diestrous interval to insure its potential duration for 3

days. All 12 rats received 50 μg. estradiol benzoate on the second day of the second diestrus as illustrated and were autopsied about 48 hours later. The vaginal smears became proestrous one day after the estrogen treatment and were fully cornified on the second day. At autopsy the only recent corpora lutea present were those representing the progesterone cycle (10 cases). The current sets of follicles gave no evidence of luteinization or preovulatory changes.

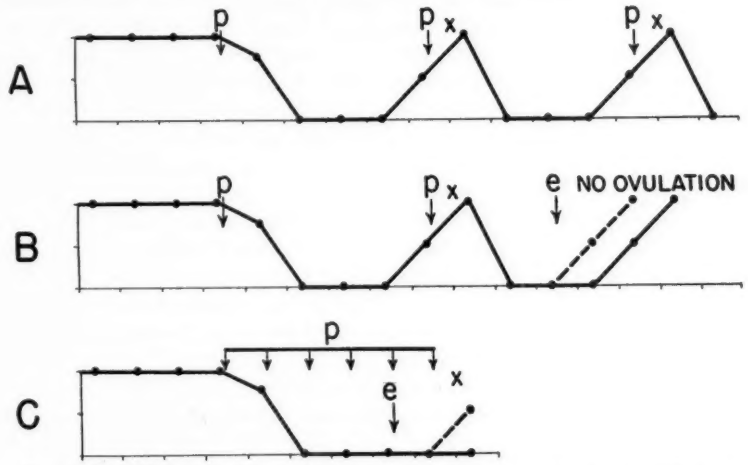


FIG. 7. Experiments with persistent-estrous DA rats. Units of the ordinate and abscissa have the same meaning as in fig. 1.

A. Standard procedure for producing "progesterone cycles," based on data reported previously (Everett, 1943). Each dose of progesterone (p) is 1.0 mg. New corpora lutea induced in about 70% of the cycles.

B. A "progesterone cycle," followed by estrogen (e) on the second day of the second diestrus. No evidence of ovulation 48 hrs. after the estrogen (contrast with figs. 1, D and 2, F).

C. A "pseudopregnancy" maintained by daily injection of progesterone (1.5 mg.). Estrogen induces ovulation in many such cases (see text).

In a correlated experiment, not illustrated, attempts were made to accelerate ovulation time in nine 5-day cyclic, young DA females by administering estrogen during diestrus. The average age was 112 days (range 87 to 147), well before the expected onset of persistent estrus, with two exceptions. Seven of the 9 rats received 50 μg. estradiol benzoate, 1 received 25 μg. and 1 received an estradiol crystal implanted subcutaneously. In every instance the estrogen was administered on the second day of diestrus and autopsy was performed 2 days later. In only 2 cases out of the 9 were tubal ova demonstrated and new corpora lutea found. The others had not ovulated and their ovaries contained sets of large follicles. This low score is highly significant when compared with the uniformly positive response obtained in the 5-day cyclic Vanderbilt rat.

Induction of ovulation by estrogen during progesterone-induced pseudopregnancy. From the several studies mentioned above and from our earlier work, it was apparent that progesterone *per se* is not incompatible with ovulation. When a rat receives 1.5 mg. of progesterone or more daily, beginning in early diestrus, a possible reason that ovulation is suppressed is that gonadotrophin (LH?) secretion is diminished to the point where little estrogen is produced or at least no sharp increments of estrogen occur. During pregnancy or pseudopregnancy induced in rats by sterile mating, it is known (Everett, 1947) that administration of estrogen causes ovulation and new corpus luteum formation within 48 hours. Although in DA rats the LH-release mechanism is so apparently refractory to estrogen, especially after reaching the state of persistent estrus, the majority of them respond, nevertheless, by forming new corpora lutea if progesterone is given when estrogen levels are rising (fig. 7, A). It seemed probable that if pseudopregnancies were established in persistent-estrous DA rats by daily injection of progesterone, ovulation and luteinization could then be induced by injection of estrogen. This experiment was performed on 10 rats, as illustrated in figure 7, C. Each animal received 1.5 mg. of progesterone daily for 6 days. On the fifth day each rat also received 50 μg. of estradiol benzoate. Autopsy was performed on the second day after the estrogen therapy. Ovulation had occurred in 4 of the rats, tubal ova and new corpora lutea being demonstrated. The ovaries of 3 others contained numerous luteinizing follicles which had failed to rupture. The remaining 3 rats had not ovulated, their ovaries containing sets of follicles which were not hyperemic. Histologically, these latter follicles gave no evidence of preovulatory swelling. Thus, positive responses may be claimed for 7 of the 10 rats and even the 4 cases of proven ovulation are highly significant in view of the extreme refractoriness of such rats to estrogen under other circumstances.

DISCUSSION

In light of the above evidence the theory that progesterone action on the hypophysis is merely to suppress LH secretion (and hence to suppress ovulation) appears no longer tenable. Under certain conditions progesterone clearly facilitates the release of luteinizing hormone. Examination of the conditions under which the respective actions occur may assist in resolving the seeming paradox.

Suppression of estrous changes, with postponement of ovulation, occurs when progesterone acts early in the cycle, beginning no later than 2 days before the next expected proestrus (Phillips, 1937; and the above data). At such times estrogen secretion is probably slight. Furthermore, the hypophysis has recently lost much of its LH. There is at present no clear evidence to show whether the LH content remains low during continued action of progesterone. Evans and Simp-

son (1929) found a slight increase of gonadotrophic potency in hypophyses of pregnant rats when compared with glands from the nonpregnant females. Siegert (1933), however, reported a lower potency during pregnancy then during diestrus. The fact that after a regime of progesterone injections (as in fig. 2, C) a measured interval of about 3 days is required for the spontaneous reappearance of proestrous changes, suggests that during the period of action of progesterone the actual LH content of the gland continues at low level. This does not deny continued synthesis of LH. Perhaps under these circumstances it is released as rapidly as it forms and thus never accumulates in quantity sufficient to precipitate ovulation.

A close study of the experiments represented in figures 2, C, and 2, E, will make evident the wide difference of response which results from a relatively small difference in timing of the second injection of progesterone. If after the first injection the second one follows in about 24 hours, the impending estrus and ovulation are retarded an additional 24 hours. If, however, after the first injection one waits 48 hours to give the second one, the impending estrus and ovulation are accelerated. In the former instance the conditions are those discussed in the preceding paragraph. In the latter, by allowing the extra day to intervene, these conditions have changed. We may legitimately believe that on the third day of diestrus estrogen secretion is now considerably elevated and an increased store of LH appears likely. Under such circumstances, either in the retarded 4-day cycle (fig. 2, E) or in the natural 5-day cycle, progesterone facilitates ovulation. In the normal 4-day cycle, however, when progesterone is given 1 day before expected proestrus no facilitation is obtained (fig. 2, B). In some manner this failure is a function of the shorter interval after the last estrus and the last ovulation. It is reasonable to think that here estrogen secretion is still rather slight (at the time of progesterone injection) and also that elaboration of a store of hypophyseal LH has not yet progressed in adequate degree.

One may suggest that in the phenomena in which progesterone facilitates ovulation estrogen is the primary underlying factor, progesterone acting as a modifier. Hohlweg and Dohrn (1931) reported that while a certain minute quantity of estrogen (1 R.U. of Progynon) is inadequate by itself for prevention of castration-cell formation in the rat hypophysis, the same amount combined with 1 RBT. U. of progestin is entirely sufficient. It is also known that progesterone exerts a "sparing action" upon estrogen (Smith and Smith, 1946), preventing destruction of estrone by the liver. Such action may explain the Hohlweg and Dohrn observation. It may also account for the facilitation of ovulation and acceleration of vaginal cornification by progesterone. There are certain considerations, however, which make it difficult to accept such an explanation outright.

In the first place, it is not yet certain whether estrogens themselves

are responsible for the various actions upon the hypophysis such as LH release, or whether break-down products, such as the lactone suggested by the Smiths, are truly the active agents. If it is such "inactivation" products which are effective, then by preventing inactivation progesterone ought to prevent rather than foster stimulation of the hypophysis. It should be noted that Bradbury (1947) reports failure of two samples of Westerfeld's lactone to produce effects upon the immature rat hypophysis like those induced by active estrogens.

In the second place, it is probable that the stimulus to release of the ovulatory surge of LH occurs within a very few hours after injection of progesterone. Ovulation time is estimated as roughly 15 hours after injection. Judging from data on the rabbit (Fee and Parkes, 1928; Waterman, 1943, and refs. cited there) luteinizing hormone must usually be released in that species 9 to 10 hours before ovulation occurs. In the cyclic rat, it appears that neurohumoral stimulation essential for LH release passes to the hypophysis between 9 and 11 hours before ovulation time (Everett, Sawyer and Markee, 1948). If a similar interval between LH release and ovulation time occurs in the rat after progesterone injection, then the stimulation of the hypophysis must be accomplished within some 5 or 6 hours after administration of the hormone. Thus, if progesterone acts here by preventing estrogen inactivation by the liver, we must imagine the latter steroid(s) to be accumulating at very rapid rate, until a sufficiently high blood estrogen level is attained to stimulate the LH-release mechanism. This or an analogous indirect process may actually occur, but it seems equally plausible that progesterone modifies estrogenic stimulation of the hypophysis more directly, perhaps by lowering the threshold of the LH-release mechanism to estrogen. The experiment with persistent-estrous rats cited above (fig. 7, C) should be recalled, in which it was shown that progesterone therapy for several days renders many of these animals no longer refractory to estrogen.

The assumption in the above paragraphs that progesterone acts through the hypophysis, rather than upon the ovary, is well founded. The evidence in brief is as follows: (1) Induction of ovulation by progesterone in persistent-estrous rats is asccompanied by "repair" of the interstitial tissue (Everett, 1940, 1941, 1943). (2) Although not cited in the present report, induction of 'premature' ovulation in 5-day cyclic rats is accompanied by depletion of cholesterol in the interstitial tissue, an indication of LH action (cf. Claesson and Hillarp,1947), and by increased cholesterol storage in the corpora lutea of the preceding set, a further function of increased LH secretion (Everett, 1947). (3) Induction of 'premature' ovulation in the 5-day cyclic rat by progesterone may be blocked by administration of either dibenamine or atropine in large doses (Everett and Sawyer, unpublished; cf. Sawyer, Everett and Markee, 1948; Everett, Sawyer and Markee, 1948). Since the studies with these drugs in the rabbit

(Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1948) clearly imply that they prevent ovulation by blocking neurohumoral stimulation of the anterior lobe (Markee, Sawyer and Hollinshead, 1948), it is most logical to conclude that in the rat progesterone under proper conditions somehow enhances such stimulation.

The acceleration of vaginal cornification by progesterone is a puzzling question. Jones and Astwood (1942), in a detailed study of the responses of primed castrate rats to estrogen and to various combinations of estrogen and progesterone, reported that progesterone causes neither acceleration nor retardation of the vaginal estrous changes during the first 3 to 4 days of treatment. Furthermore, they reported and it is generally recognized (cf. Gustavson, 1939), that a delay of 36–48 hours occurs between administration of estrogen and the onset of distinct vaginal estrous changes. The observed response to progesterone in the 5-day cyclic rat is a far more rapid phenomenon. Furthermore, it is prevented by Dibenamine or atropine treatment (Everett and Sawyer, unpublished) and, hence, is probably caused indirectly by an hypophyseal secretion (LH?). We must, then, conceive of a substance which is secreted by the ovaries(?) some hours after progesterone injection and which acts on the vagina much more rapidly than the estrogens with which we are familiar.

The ability of pregesterone to foster ovulation appears at the present writing to result from an enhancement of the action of estrogen. Bradbury's (1947) observation, that in immature rats treated with estrogen the concomitant action of progesterone prevents discharge of gonadotrophin, may appear to controvert such an argument. The conditions in his experiments were, however, distinctly different than those reported herewith. His conclusions cannot legitimately be construed to mean that progesterone always prevents release of gonadotrophin. Neither can the evidence obtained in the rabbit be so construed, for in those instances progesterone was administered for several days preceding copulation (Makepeace, Weinstein and Friedman, 1937) and 24 to 72 hours before administration of copper acetate (Friedman, 1941). Against this evidence should be considered more recent information reported by Klein and Mayer (1946; Klein, 1947). It is known that rabbits will copulate while pregnant, but that ovulation does not then occur (Hammond and Marshall, 1925). However, Klein and Mayer found that when pseudopregnant or pregnant rabbits were treated with estrogen and then mated, new ovulation occurred and a second set of corpora lutea was formed. This is a very significant observation if it can be confirmed—for two reasons. It indicates that estrogen favorably affects release of LH in a species in which such effects have been repeatedly denied. Of more importance for our present argument, it suggests that progesterone suppresses ovulation in the rabbit not directly, but by indirectly lowering

estrogen secretion. When extrinsic estrogen is supplied, progesterone is compatible with release of the luteinizing hormone.

The ideas expressed in this discussion require most cautious evaluation before final conclusions are reached. There can, however, be no doubt that progesterone acts upon the hypophysis in two seemingly different ways depending on other conditions. Whether, in species other than the rat, progesterone actually facilitates release of LH remains an open question.

SUMMARY

In normal rats with predictably regular 4-day and 5-day cycles, respectively, a series of experiments is described, whereby ovulation time and other features of the estrous cycle may be either accelerated or retarded. In the normal 5-day cycle, injection of 1 to 2 mg. of progesterone on the third day of diestrus accelerates ovulation and vaginal cornification about 24 hours. Equivalent results follow administration of certain estrogens on the second day of diestrus. Progesterone does not accelerate the 4-day cycle when injected on the final (second) day of diestrus.

The 4-day cycle is retarded 1 day by injection of 1.5 mg. of progesterone on the first day of diestrus, becoming thus a 5-day cycle. (Continued daily injection of this dose for n days retards ovulation n days.) In such an artificial 5-day cycle, injection of progesterone on the third day of diestrus, omitting treatment on the second day, accelerates the expected ovulation and vaginal changes about 24 hours, just as it does in the normal 5-day cycle. Similarly, in the artificial 5-day cycle, treatment with estrogen on the second day of diestrus accelerates ovulation.

Persistent-estrous rats of the defective DA strain are refractory to estrogen in the respect that ovulation and luteinization cannot be induced in them by estrogen, even when treated during the diestrous intervals resulting from earlier progesterone treatment. However, *during* pseudopregnancies induced in such animals by daily injection of progesterone (1.5 mg.), injection of estrogen has produced ovulation in a significant number of cases.

Thus progesterone "suppresses" ovulation and other features of estrus only under certain conditions. When estrogen levels are elevated progesterone facilitates ovulation, at least in the rat. This it may accomplish by modification of the threshold of the LH-release mechanism to estrogen or perhaps by modification of estrogen metabolism.

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REFERENCES

ASTWOOD, E. B., AND H. L. FEVOLD: *Am. J. Physiol.* 127: 192. 1939.
BOLING, J. L.: *Anat. Rec.* 82: 131. 1942.
BRADBURY, J. T.: *Endocrinology* 41: 501. 1947.
BURDICK, H. O., AND R. WHITNEY: *Am. J. Physiol.* 132: 405. 1941.
CLAESSON, L., AND N.-Å. HILLARP: *Acta physiol. Scandinav.* 14: 102. 1947.
DEMPSEY, E. W.: *Am. J. Physiol.* 120: 126. 1937.
DURY, A., AND J. T. BRADBURY: *Am. J. Physiol.* 139: 135. 1943.
EVANS, H. M., AND M. E. SIMPSON: *Am. J. Physiol.* 89: 379. 1929.
EVERETT, J. W.: *Endocrinology* 25: 123. 1939.
EVERETT, J. W.: *Endocrinology* 27: 681. 1940.
EVERETT, J. W.: *Anat. Rec.* 79 (No. 3, Suppl.): 87. 1941.
EVERETT, J. W.: *Anat. Rec.* 82: 409. 1942.
EVERETT, J. W.: *Endocrinology* 32: 285. 1943.
EVERETT, J. W.: *Endocrinology* 34: 136. 1944.
EVERETT, J. W.: *Anat. Rec.* 94: 517. 1946.
EVERETT, J. W.: *Endocrinology* 41: 364. 1947.
EVERETT, J. W., C. H. SAWYER AND J. E. MARKEE: *Anat. Rec.* 100: 740. 1948.
FEE, A. R., AND A. S. PARKES: *J. Physiol.* 67: 383. 1929.
FEVOLD, H. L., F. L. HISAW AND R. O. GREEP: *Am. J. Physiol.* 114: 508. 1936.
FRIEDMAN, M. H.: *Endocrinology* 29: 628. 1941.
GUSTAVSON, R. G.: Bioassay of androgens and estrogens, in: Edgar Allen: *Sex and Internal Secretions*, 2nd ed., Chap. XIV. The Williams and Wilkins Co., Baltimore. 1939.
HAMMOND, J., AND F. H. A. MARSHALL: *Reproduction in the rabbit.* Oliver and Boyd, Edinburgh. 1925.
HISAW, F. L.: *Physiol. Rev.* 27: 95. 1947.
HOHLWEG, W.: *Klin. Wchnschr.* 13: 92. 1934.
HOHLWEG, W., AND M. DOHRN: *Wien. Arch. f. inn. Med.* 21: 337. 1931.
HOHLWEG, W., AND A. CHAMORRO: *Klin. Wchnschr.* 16: 196. 1937.
JONES, G. E. S., AND E. B. ASTWOOD: *Endocrinology* 30: 295. 1942.
KLEIN, M.: *J. Endocrinol.* 5 (Proc. Soc. Endocrinol.) XXV. 1947.
KLEIN, M., AND G. MAYER: *Compt. rend. Soc. de biol.* 140: 600. 1946.
LANE, C. E.: *Am. J. Physiol.* 110: 681. 1935.
MAKEPEACE, A. W., G. L. WEINSTEIN AND M. H. FRIEDMAN: *Am. J. Physiol.* 119: 512. 1937.
MARKEE, J. E., C. H. SAWYER AND W. H. HOLLINSHEAD: *Recent Progress in Hormone Research* 2: 117. 1948.
PHILLIPS, W. A.: *Am. J. Physiol.* 119: 623. 1937.
SAWYER, C. H., AND J. W. EVERETT: *Endocrinology* 39: 307. 1946.
SAWYER, C. H., J. W. EVERETT AND J. E. MARKEE: *Anat. Rec.* 100: 774. 1948.
SAWYER, C. H., J. E. MARKEE AND W. H. HOLLINSHEAD: *Endocrinology* 41: 395. 1947.
SAWYER, C. H., J. E. MARKEE AND B. F. TOWNSEND: In press 1948.
SELYE, H., J. S. L. BROWNE AND J. B. COLLIP: *Proc. Soc. Exper. Biol. & Med.* 34: 472. 1936.
SIEGERT, F.: *Klin. Wchnschr.* 12: 145. 1933.
SMITH, O. W., AND G. V. SMITH: *J. Clin. Endocrinol.* 6: 483. 1946.
WATERMAN, A. J.: *Am. J. Anat.* 72: 473. 1943.
WESTMAN, A., AND D. JACOBSOHN: *Acta obst. et gynec. Scandinav.* 18: 115. 1938.

BIO-ASSAY OF CALORIGENIC SUBSTANCES USING TADPOLES OF XENOPUS LAEVIS[1]

C. F. HAMILTON, A. ALBERT AND MARSCHELLE H. POWER
Mayo Foundation and Mayo Clinic
ROCHESTER, MINNESOTA

THE ASSAY of thyroidal and other calorigenic preparations in amphibia has not been completely satisfactory. Recently Deanesly and Parkes (1945 a, b) have devised a new method for thyroidal assay utilizing the eruption of the fore limbs in tadpoles of Xenopus laevis. The many obvious advantages of their method may establish it as the most useful procedure for work in this field. However, the time required for the assay (seven days) and the relative insensitivity of the test are disadvantages for certain special problems involving the thyroid. By using the appearance of the fore limb buds as an endpoint we have been able to modify the procedure so that the time required has been shortened from seven to three days and the sensitivity has been increased about twentyfold.

MATERIALS AND METHODS

The details of the care, handling and feeding of the adult toads were essentially those described by Deanesly and Parkes. Mating of the adults was secured by injecting 250 and 500 I. U. of chorionic gonadotrophin into the male and female Xenopus respectively. A number of commercial preparations, such as korotrin (Winthrop Chemical Co., Inc.) and antuitrin S (Parke, Davis & Company), were used with equal satisfaction. Five batches of tadpoles, varying in number from several hundred to several thousand per batch, were obtained. A week after hatching, the tadpoles were fed liver powder (Armour Laboratories) at a dose of 1 mg. per tadpole per day. The tadpoles were maintained at room temperature (there being no facilities to maintain constant temperature during growth). Within fourteen to twenty-one days they had grown to an optimal size (16 to 24 mm. length) for use. The animals selected for this length were then distributed among 250 cc. beakers, each containing 5 tadpoles in 200 cc. of tap water.

A variety of calorigenic and noncalorigenic substances were tested (table 1). Insoluble substances were passed through a 100 mesh screen and added to the water as a fine suspension. Those compounds readily soluble in water were added in solution. The beakers were then placed in the incubator at $25° \pm 1°$ C. A range of five or six dosage levels separated at log dose intervals

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TABLE 1. SUBSTANCES ASSAYED FOR METAMORPHIC ACTIVITY

Materials	Range of dosage, micrograms	Iodine,* per cent	Thyroxine,* per cent
dl-Thyroxine	0.1–3.0	65.4	100
Desiccated thyroid†	10–1,000	1.0	0.4
Protamone‡	10–1,000	6.4	3.3
Iodocasein§	10–1,000	6.2	2.6
Radioactive iodocasein§	10–1,000	5.3	2.0
Iodine	1,000–100,000	100	0
Potassium iodide	1,000–100,000	75.9	0
l-Tyrosine	100–100,000	0	0
Casein	100–100,000	0	0
3–5 l-Diiodotyrosine	1,000–100,000	58.6	0

* Analytic methods to be described elsewhere.
† Strong (Parke, Davis & Company, Detroit, Michigan).
‡ Cerophyll Laboratories, Kansas City, Missouri. Protamone is a commercial iodocasein preparation. The trade name is purposely used to distinguish it from our own preparations of iodocasein.
§ Our own preparations, to be described elsewhere.

of about 0.5, plus a set of 20 animals as controls, was usually employed for each assay. Since 10 to 20 animals were tested at each dosage of any material the final assay represented a determination based on from 50 to 120 tadpoles. Some twenty-two assays on a little more than 2,000 animals were carried out.

After three days, the animals were examined under a hand lens and the percentage of animals showing development of the fore limb buds was determined. The fore limb buds did not appear naturally in normal development until after the first month under the conditions described. Active thyroidal preparations induced a rapid development of the limb buds which could be seen quite easily as grayish white bars extending laterally. After the third day the procedure outlined by Deanesly and Parkes was followed in exact detail in order to compare our modification with the original method, since the latter method at this point consists only in changing the water at the third day and determining the number of animals showing eruption of the fore limb buds at the end of seven days.

For analysis of the dose response relation, the percentages of animals showing a positive response were converted into empirical probits and plotted against the logarithm of the dose. The intercept of the line corresponding to a probit of 5 was designated as the median effective dose (M.E.D.). Although this method of calculation is not necessarily a complete method or the most appropriate method of dose response analysis, it has been utilized so that our results could be directly compared with those of Deanesly and Parkes. The M.E.D.'s, thus obtained, represent the dose of substance per 200 cc. of water containing 5 tadpoles and is not to be construed as representing the effective dose per tadpole.

RESULTS

1. Preliminary Experiments.—Several sighting tests were performed with iodocasein according to the method of Deanesly and Parkes. The M.E.D.'s obtained fell between 450 and 550 micrograms, close to the average range of 460 to 660γ found by the foregoing authors. It was noted however, that many animals on lower dosages did not erupt their fore legs but showed accelerated fore limb development which stopped short of eruption. Moreover, the accelerated fore limb development could be seen as early as one or two days after

TABLE 2. THE COURSE OF THE FORMATION AND ERUPTION OF THE FORE LIMBS AT VARIOUS DOSAGES OF CALORIGENIC SUBSTANCES

Materials tested	Dosage, micrograms	Percentage of animals showing "bars" at daily intervals							Percentage of animals showing eruption at daily intervals			
		1	2	3	4	5	6	7	1–4	5	6	7
Protamone	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	5	5	5	30	30	0	0	0	0
	25	0	20	25	50	60	80	90	0	0	0	0
	50	20	50	85	90	100	100	100	0	0	0	0
	100	20	60	100	100	100	100	100	0	0	10	10
	250	20	75	100	100	100	100	100	0	0	10	25
	500	30	80	100	100	100	100	100	0	20	40	55
Iodocasein	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	10	20	0	0	0	0
	25	0	0	10	10	10	15	25	0	0	0	0
	50	0	15	15	15	40	40	40	0	0	0	0
	100	10	26	52	52	60	60	70	0	0	0	0
	250	10	35	80	94	100	100	100	0	0	5	5
	500	25	65	100	100	100	100	100	0	25	45	58
Desiccated thyroid	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	5	25	25	53	53	0	0	0	0
	50	0	15	70	80	80	85	85	0	0	0	0
	100	0	33	74	100	100	100	100	0	0	0	0
	250	0	25	85	100	100	100	100	0	0	0	0
	500	0	25	89	100	100	100	100	0	10	16	16
	1,000	0	25	100	100	100	100	100	0	10	25	25
	5,000	15	50	100	100	100	100	100	0	100	100	100

administration of the dose. These two observations led to the thought that accelerated fore limb differentiation could be used as an endpoint, thus shortening the assay and greatly increasing its sensitivity. It became necessary therefore to investigate the temporal sequence of the fore limb differentiation, to analyze the dose-response curves and to study the specificity of the proposed reaction.

2. The Course of the Formation and Eruption of the Fore Limb Buds.—A sample of the results obtained at different dosage levels for iodoproteins at daily intervals after administration is shown in table 2. The percentage of animals showing the appearance of the forelimb buds (hereafter referred to as "bars") and the percentage of animals showing eruption of the fore limb ("erupters") were determined. When the results shown in table 2 were plotted against time, it was more easily seen that the rate of response at all doses for bars was most rapid during the first three days. After the third day the rate of response declined. In other assays, not detailed here, the proportion of positive responses increased but slightly after the third day

and in some experiments asymptotic values were reached. In any case, the third day represented the most appropriate time for reading the end-point, since at this time there was the greatest spread among the effects of various doses and since the rate of response either declined appreciably or became asymptotic after this time. Eruption of the fore limbs did not take place until after the fourth day since they were differentiating up to this time. When the number of animals showing eruption was plotted, the temporal sequence was found to be essentially that previously described. It was also interesting that the time curves of eruption paralleled those observed for bars. Inspection of table 2 will indicate the increased sensitivity conferred by using bars as end-point.

3. Quantitative Aspects.—The assays obtained are shown in figure 1, where the percentage of response was transformed into probits and plotted versus the logarithm of the dosage. Since each batch of

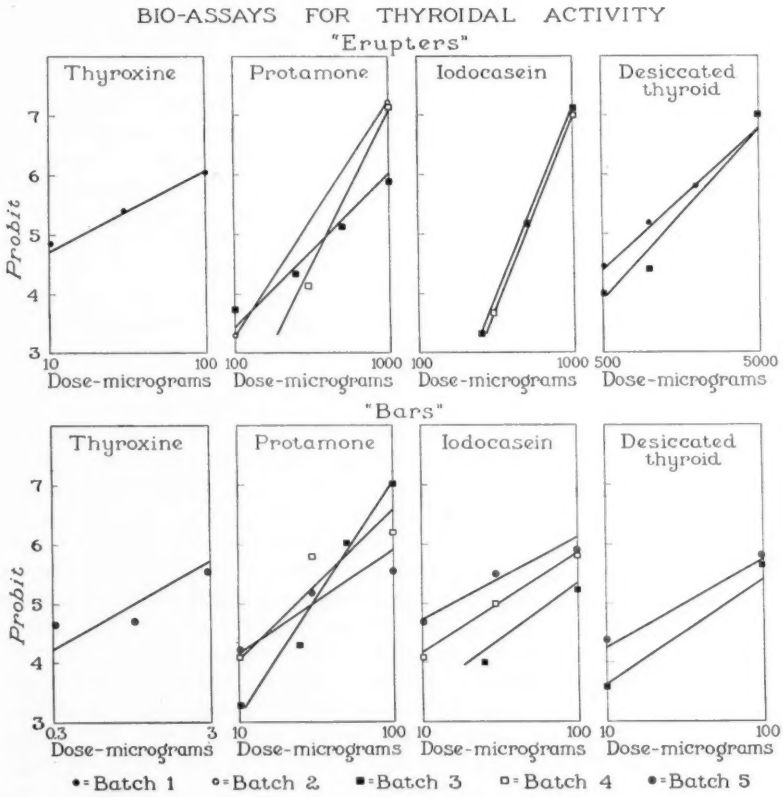

FIG. 1. The dose-response relationship of various calorigenic materials.

tadpoles may show different sensitivity, the individual assays are distinguishable by batch number as well as by material tested. The slopes of the assays using erupters varied from 2.8 to 6.6, a variation that was also encountered in the work of Deanesly and Parkes. Using bars as end-point, the slopes varied from 1.4 to 3.9, apparently somewhat lower values. However, sufficient data are not yet available to indicate whether or not the slopes for bars are generally lower and hence to signify a somewhat lower precision. More satisfactory slopes (that is, greater than 5) presumably can be obtained by further investigation of the dose response curves.

The median effective doses of the various materials are shown in table 3. The results (erupters) compare favorably with those reported for similar substances by the British workers. The ratios of activity

TABLE 3. MEDIAN EFFECTIVE DOSES (MICROGRAMS) OF CALORIGENIC SUBSTANCES

Tadpole batch number	End-point: Fore limb eruption				End-point: Appearance of bars			
	Protamone	Iodocasein	Thyroid	Thyroxine	Protamone	Iodocasein	Thyroid	Thyroxine
1			900	16				
2	270							
3	400	480	1,020		30	60	60	
4	400	480			24	30		
5					30	15	32	1
Average	357	480	960	16	28	35	46	1
Ratio (thyroxine =1)	22	30	60	1	28	35	46	1

for most of the preparations (using thyroxine as 1) were roughly of the same order of magnitude when the two end-points were compared. It can be seen from table 3 that an over-all average increase of sensitivity of about twentyfold was achieved. Not shown in table 3 are the results for radioactive iodocasein, tested to assure its biologic potency for use in investigations on the metabolism of iodocasein in human beings. This preparation proved to be one of our best iodocaseins since its M.E.D. was 7γ (bar end-point).

4. Specificity.—It has been held by some that the metamorphic changes in amphibia are not specific reactions to thyroidal substances, since materials like iodine and diiodotyrosine also induce such changes. Reineke and Turner (1942), and Deanesly and Parkes (1945 a, b), however, have felt their assays to be highly specific. We are in agreement with the latter opinion for several reasons, the most pointed of which is the available data that miscellaneous substances under the conditions of the test have been found to be inert. Seaweed, potassium iodide, dichlorotyrosine and dibromotyrosine were found by Deanesly and Parkes to be inert at 10 mg. dose levels. Our results show that such additional substances as molecular iodine, tyrosine and casein

(table 1) were inert at high dosage levels when tested by both methods of assay. It should be pointed out that the dose levels shown in table 1 represent maximal tolerated amounts, for the next highest dose for many of the compounds proved lethal. The only "miscellaneous" substance with slight thryoidal activity was diiodotyrosine. However, the M.E.D. (bars) of this substance was 8.6 mg. or some 6,000 times greater than that of thyroxine run simultaneously. On the basis of the erupter end-point, it was between 600 and 800 times greater than that of thyroxine. Since in routine assay of calorigenic proteins the dosage does not usually exceed 1 mg., it would be quite impossible for any contaminating diiodotyrosine to interfere with the assay or to contribute through its thyroidal action per se to the result to the extent of more than a few per cent. In the absence of evidence to the contrary, it appears likely that the assay of thyroidal materials in amphibia is quite specific under the given conditions of the assay.

DISCUSSION

Although the procedure and the results of the modified bio-assay merit little further discussion, the general interpretation of bio-assays for thyroidal activity using amphibia deserves some comment. Since it is not known precisely to what extent such assays can be applied to mammalian forms, particularly to human beings, the interpretation of results obtained in amphibia may be somewhat hazardous. It is probable that the same calorigenic substances can be utilized quite differently by different organisms and therefore mutual interconversion of values would not be possible. The difference in activity of d-thyroxine and l-thyroxine in various organisms is a case in point. There is, therefore, need in other classes of vertebrates for bio-assay methods similar in convenience and precision to those available for amphibia.

When amphibia alone are used , a number of problems as yet unsolved must be considered. The foremost of these problems is the decision as to which of the many active materials is to be used as the standard of reference for thyroidal activity. Were the effect of all active substances directly proportional to their thyroxine content, a standard reference of thyroxine = 1 could always be employed. Our results, however, would lead us to conclude that the activity of all preparations was not proportional to their thyroxine content. In table 1 the thyroxine content of the various preparations is given. From these data M.E.D.'s (table 3) of the various compounds can be calculated in terms of their thyroxine content. Thus the M.E.D., using the erupter end-point was 16γ for thyroxine, 12γ for protamone, 12γ for iodocasein and 4γ for desiccated thyroid. Using the bars end-point the M.E.D. was 1γ for thyroxine, 0.9γ for protamone, 0.9γ for iodocasein and 0.18γ for desiccated thyroid. While the activity of the two preparations of iodocasein would thus appear to be roughly proportional

to their thyroxine content, the activity of desiccated thyroid was four or more times greater than its thyroxine content would allow.

Data of similar nature in terms of acid-insoluble iodine of a great variety of iodinated proteins (ardein, plasma, casein) prepared by Rivers and Randall (1945) have been reported (Deanesly and Parkes, 1945 b). Whether or not acid-insoluble iodine represents thyroxine iodine is problematical. On the assumption that it does, however, the activity of some of the better iodocasein preparations would be only twice as much as their thyroxine content would indicate. Desiccated thyroid was estimated to be some ten to twenty times more potent than iodocasein, since iodocasein containing 1 to 2 per cent of acid-insoluble iodine gave the same physiologic effect as desiccated thyroid containing 0.1 per cent of acid-insoluble iodine. Our results using a more direct determination of thyroxine are in fairly good agreement with the foregoing. The greater activity of desiccated thyroid over artificial iodoproteins in terms of thyroxine or acid-insoluble iodine appears to hold true not only for amphibia but also for birds and mammals.

A different relationship had been observed previously by Reineke and Turner, who found that the activity of iodocasein in Rana tadpoles was equal to that of thyroxine in terms of total organic iodine. Two objections to the significance of this relationship have been raised by Deanesly and Parkes: (1) that thyroxine when combined in protein (meaning, presumably, desiccated thyroid) is appreciably more effective than the same amount of pure thyroxine; (2) that a substantial proportion of organic iodine in iodoproteins is acid-soluble iodine, which is largely inert biologically. Calculation of our results in terms of organic iodine (the percentages of iodine for organic compounds in table 1 may be assumed to represent almost wholly organic iodine) reveals a good agreement between desiccated thyroid and thyroxine, but iodocasein would be about a third as active as thyroxine.

Finally, it is of interest to compare the results of the bio-assays with respect to total iodine content, since recently several aspects of the effects of elementary iodine on processes related to the thyroid have received some attention (Albert and co-workers, 1946; Dvoskin, 1947). Many years ago, Swingle (1919) reported that iodine crystals promote metamorphosis in amphibia, and recent work (Dvoskin, 1947; Nelson and Wheeler, 1948) has proved that elementary iodine produces thyroidal effects in mammals. A plausible explanation for this effect is that elementary iodine, when placed directly on or in tissues, induces a local iodination of tissue proteins, some of which are calorigenically active on absorption. Our results (table 1) do not show any relation between activity and total iodine. Furthermore, elementary iodine had no effect when administered in the manner prescribed, indicating that while iodoproteins may be formed at the surface of the

skin of tadpoles, their absorption was nil or negligible to the extent that no biologic response was obtained.

Failure to account for the physiologic activity of various thyroidal substances on some common basis poses a perplexing problem. It might be held that thyroxine as such is less effectively utilized than the same amount of thyroxine in combination with protein. This appears to be true for desiccated thyroid but it is not true, at least not to the same extent, for artificial iodoproteins. The existence of antagonistic substances in artificial iodoproteins which would account for their lesser activity or the presence of facilitating substances in natural thyroprotein which would account for its greater activity when equilibrated with respect of thyroxine content are possibilities that deserve experimental effort. Also, the chemical determination of thyroxine in proteins, the suspicion that acid-insoluble iodine may represent a great deal more than thyroxine iodine and increased precision in bio-assays merit further work.

In view of the foregoing, the present situation is such that there can be no universal standard of reference for activity. Because of the exceptional stability of thyroidal preparations, various compounds can be employed as standards, depending on the nature of the work involved. A reasonable suggestion is that substances having certain general features in common with thyroxine, such as chemical or molecular configuration, size and properties, be referred to thyroxine as a standard. Similarly, iodinated proteins can be used as their own standard. Thus a preparation of iodocasein could be distributed as a standard reference for iodocaseins and the same could be true for iodinated plasma or any other iodinated protein. Natural thyroproteins could be handled in the same manner in addition to the requirements already in force as stated in the United States Pharmacopoeia.

With the availability of convenient and reasonably precise bio-assay methods such as those described for Xenopus tadpoles, work of both practical and theoretical import should be accelerated. For routine assays of known, highly active materials, the method of Deanesly and Parkes is very satisfactory. When it is desired to assay small amounts, or especially when only small amounts of active compounds may be suspected, the supplementary method described in this paper for detection and assay may be used to advantage.

SUMMARY

A supplementary method for the bio-assay of thyroidal activity using the appearance of the fore limb buds of tadpoles (Xenopus laevis) is described. The significant attributes of this method are its speed and its sensitivity; it thus serves as a useful tool for the detection and assay of small amounts of calorigenic substances.

Various calorigenic substances did not show activity proportional

to their thyroxine content; natural thyroprotein was much more active than its thyroxine content would allow, whereas artificial iodoproteins were roughly proportional to their thyroxine content. Various standards of reference of activity were suggested for the assay of different calorigenic substances.

REFERENCES

ALBERT, ALEXANDER, R. W. RAWSON, PRISCILLA MERRILL, BEATRICE LENNON, AND CHARLOTTE RIDDELL: *J. Biol. Chem.* 166: 637. 1946.

DEANESLY, R., AND A. S. PARKES: *J. Endocrinol.* 4: 324. 1945a.

DEANESLY, R., AND A. S. PARKES: *J. Endocrinol.* 4: 356. 1945b.

DVOSKIN, SAMUEL, *Endocrinology.* 40: 334. 1947.

NELSON, W. O., AND HELEN E. WHEELER: *Anat. Rec.* 100: 67. 1948.

REINEKE, E. P., AND C. W. TURNER: Formation in Vitro of Highly Active Thyroproteins, Their Biologic Assay, and Practical Use. Columbia, Missouri, Research Bulletin 355, University of Missouri College of Agriculture, Agricultural Experiment Station, (Nov.) 1942, 88 pp.

RIVERS, ROSALIND P., AND S. S. RANDALL: *J. Endocrinol.* 4: 221. 1944–1946.

SWINGLE, W. W.: *J. Exper. Zoöl.* 27: 397. 1919.

FURTHER STUDIES ON THE PROTECTIVE POWER OF ADRENAL EXTRACT AND STEROIDS AGAINST TOXIC AGENTS

LENA A. LEWIS AND IRVINE H. PAGE

From the Research Division of the Cleveland Clinic Foundation

CLEVELAND, OHIO

IT HAS BEEN shown that the protective power of adrenal extracts and steroids against the action of a toxic agent, typhoid vaccine, in adrenalectomized rats, can be accurately measured (Lewis and Page 1946). This investigation has now been extended to determine the protective power of adrenal hormone against diphtheria, tetanus and botulinus toxins. Studies were also made to ascertain whether compound A acetate (11-dehydrocorticosterone acetate) and desoxycorticosterone acetate acting together gave greater protection against typhoid vaccine than either one alone. It also seemed of interest to determine the temporal relationship between presence of hormone and protective power against typhoid vaccine.

Because the question must inevitably arise as to whether results with vaccines or toxins in adrenalectomized animals are also related to active infections in normal animals, a study was undertaken of the protective power of adrenal extract in normal mice infected with pneumococci type I and type III.

METHODS

Male Sprague-Dawley rats were adrenalectomized and maintained on 0.9 per cent sodium chloride for drinking (Lewis and Page 1946). The minimal lethal dose (M.L.D.) of botulinus,[1] diphtheria,[2] and crystalline tetanus toxin[2] was determined on rats after they had been adrenalectomized for at least 5 days. For comparison, the M.L.D. was also determined on normal rats of like size and age. It was taken as the smallest amount killing all rats within 10 days after subcutaneous injection of botulinus toxin, 96 hours after tetanus toxin, and 72 hours after diphtheria toxin. A concentrated streptococcal toxin was also tested for ability to kill.

The protection afforded by different adrenal hormones against these toxins was studied by injecting adrenalectomized rats with the test solution (if in oil) 48 and 24 hours before 1.0 to 1.5 M.L.D. of toxin and daily thereafter. If the extract was an alcoholic-aqueous solution, injections were made twice daily (48, 40, 24 and 16 hours before the toxic agent and 0, 8, 24, 32

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[1] The botulinus toxin was prepared by Dr. W. A. Starin, Ohio State University.

[2] The concentrated diphtheria toxin and concentrated streptococcal toxin were supplied by Dr. H. D. Pierma, Lederle Laboratories, and the crystalline tetanus toxin was prepared by Dr. L. Pillemer, Western Reserve University.

. . . hours after). In testing for protective power, the period of observation after toxin injection was the same as that used in determining the M.L.D.

Rats were treated with compound A acetate and desoxycorticosterone acetate to determine whether synergism occurred in the protective effect against typhoid vaccine. The testing procedure we formerly employed (Lewis and Page 1946) was used and the amount of hormone in oil given per day was: 0.2 mg. compound A acetate, 0.5 mg. desoxycorticosterone acetate, 0.2 mg. compound A acetate and 0.5 mg. desoxycorticosterone acetate, 0.1 mg. compound A acetate, 0.1 mg. compound A acetate and 0.5 mg. desoxycorticosterone acetate.

The temporal relationship between presence of hormone and protective power was studied by giving adrenal extract only at the time of, or after injection of 1.33 M.L.D. typhoid vaccine. One or two toxic protection units (Lewis and Page, 1946) of adrenal extract (aqueous-alcohol) were injected 0, 30 or 60 minutes and 8 hours after vaccine. The number of rats surviving 24 hours after vaccine administration was noted.

The relative protective power of desoxycorticosterone acetate in oil solution given subcutaneously, and in aqueous solution given intravenously or intraperitoneally, one hour before injection of typhoid vaccine, was determined.

Normal mice were treated with adrenal extract and a lethal amount of typhoid vaccine given, to ascertain whether protection was secured. It was found desirable to give two injections of 1.0 ml. typhoid vaccine 16 hours apart to secure uniform lethal effect. Twenty of the mice received one half ml. extract at the time of the two vaccine injections and 8 hours after the second, and 20 received one half ml. extract 32 and 24 hours before the vaccine injections and at the time of the vaccine. Twenty mice received only typhoid vaccine.

To determine whether adrenal extract afforded protection against pneumococci type I and type III, 46 normal female mice weighing 20 to 22 grams were injected intraperitoneally with type I pneumococci and a similar number with type III. An amount was injected which had been found lethal within 96 hours to all animals. Twenty-three of each group were injected twice daily with 0.2 ml. adrenal extract and 23 with 0.2 ml. saline.

RESULTS

The minimal lethal dose (M.L.D.) of botulinus toxin was 0.2 ml. of a 1:100 solution for adrenalectomized and 0.9 ml. for normal rats (chart 1). After 2.2 M.L.D. (determined on normal rats) of toxin, 100 per cent of the animals in both groups died within 24 hours. Such uniformity in speed of lethal action was not observed, however, after smaller amounts of toxin. After 1.0 M.L.D. (determined on adrenalectomized rats) of toxin, the time of death of the adrenalectomized animals varied greatly. Twenty-five per cent died between the 48th and 72nd hour, while a like number succumbed between the 192nd and 216th hour. After 3.75 M.L.D. (determined on adrenalectomized rats) of toxin, such varied death rate was not observed. The dose-response curve was similar to that obtained on normal rats after 1.0 M.L.D.

(determined on normal rats). One hundred per cent of normal rats died between the 24th and 96th hour after 1.0 M.L.D. toxin.

Both adrenalectomized and normal rats showed a serious reduction in food intake and weight loss after injection of toxin. The partial starvation was probably an important factor in causing death of the adrenalectomized animals several days after toxin injection. Normal animals could withstand this additional stress. Ten to 12 days after receiving toxin, surviving animals resumed normal food consumption and showed rapid weight gain.

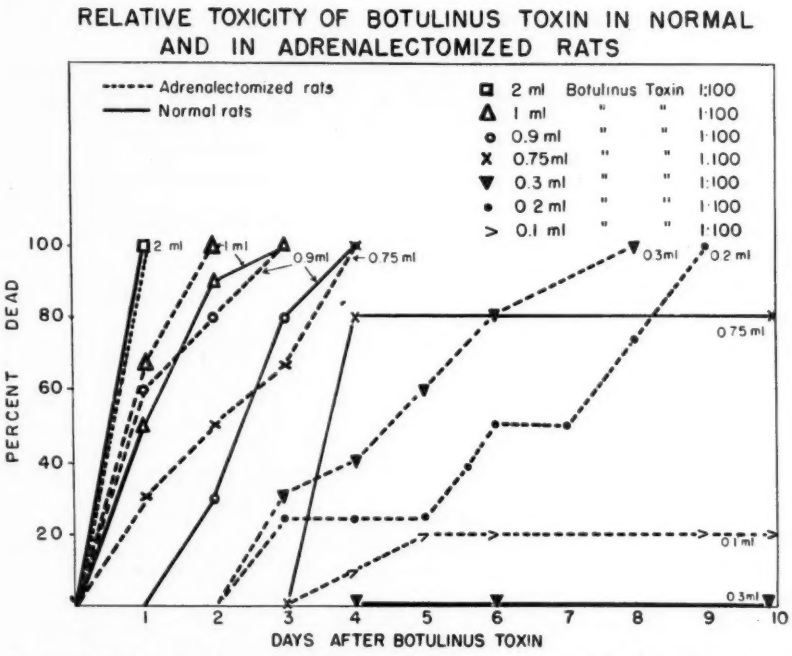

CHART 1. Relative toxicity of botulinus toxin in normal rats and in adrenalectomized rats receiving 0.9 per cent sodium chloride solution for drinking.

Ninety percent of adrenalectomized rats receiving 1.5 ml. adrenal extract were protected against 1.5 M.L.D. (determined on adrenalectomized rats) of botulinus toxin, while only 15 per cent receiving 2.0 mg. desoxycorticosterone acetate survived. The gluconeogenic action of the adrenal extract was probably important in its protective action against the toxin.

As had been found for botulinus toxin, the minimal lethal dose of diphtheria toxin was greater for normal than for adrenalectomized rats. The M.L.D. for adrenalectomized rats was 0.2 ml., and for normal rats 1.0 ml. Adrenal extract (1.5 ml. per day) protected adrenalec-

tomized rats against as much as 1.5 M.L.D. diphtheria toxin (M.L.D. determined on adrenalectomized rats). Forty per cent of adrenalectomized animals receiving 0.4 mg. compound A acetate per day were protected against 1.5 M.L.D. toxin, while one or two mg. of desoxycorticosterone acetate per day was ineffective.

In contrast with the differences in toxicity of botulinus and diphtheria toxins for normal and adrenalectomized rats, the M.L.D. of crystalline tetanus toxin was 60 mouse M.L.D. for both groups. One mouse M.L.D. is contained in 0.000013 gamma crystalline tetanus toxin nitrogen (Pillemer and Wartman 1947). The speed of action of toxin in normal and adrenalectomized groups was the same and varied

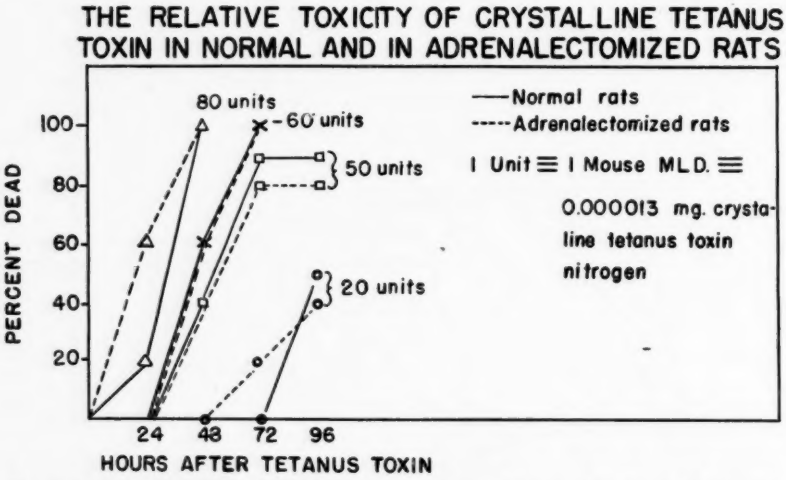

CHART 2. Relative toxicity of crystalline tetanus toxin in normal rats and in adrenalectomized rats receiving 0.9 per cent sodium chloride solution for drinking.

directly with the dose (Chart 2). One and a half ml. of adrenal extract (Upjohn) per day or 2 mg. desoxycorticosterone acetate per day had no protective effective against its lethal action in adrenalectomized rats.

Injection of as much as 2.5 ml. of concentrated streptococcal toxin into adrenalectomized or normal rats produced no evident toxic effects. It appears that both normal and adrenalectomized rats have great natural resistance to this type of toxin.

Greater protection of adrenalectomized rats against 1.33 M.L.D. typhoid vaccine was secured when small amounts of compound A acetate and desoxycorticosterone acetate were given at the same time than by either steroid in similar dosage alone. When 0.5 mg. desoxycorticosterone acetate and 0.1 mg. compound A acetate in oil were given 48 and 24 hours before and at the time of administration of typhoid vaccine, 74 per cent of the rats survived. None of those re-

ceiving only 0.1 mg. compound A acetate were alive 24 hours after injection of typhoid vaccine and only 33 per cent receiving 0.5 mg. desoxycorticosterone acetate were protected. All rats receiving 0.2 mg. compound A acetate and 0.5 mg. desoxycorticosterone acetate survived, while 60 per cent receiving 0.2 mg. compound a acetate alone were protected.

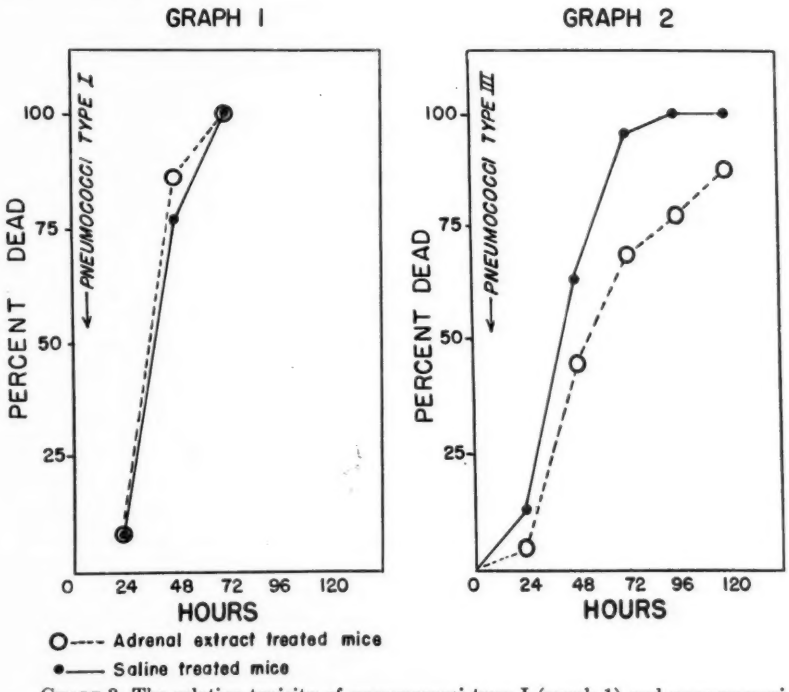

CHART 3. The relative toxicity of pneumococci type I (graph 1) and pneumococci type III (graph 2) in normal mice receiving injections of 0.2 ml. adrenal extract or 0.2 ml. 0.9 per cent sodium chloride solution, twice daily.

Eighty per cent survival occurred in adrenalectomized rats which received no priming with hormone before the typhoid vaccine, but had received one or two toxic protection units of adrenal extract either at the time of its injection or 30 minutes later. Repetition of the dose 8 hours later, as in the standard procedure, was necessary to attain this survival rate. No greater protection was secured with two toxic protection units per dose than with only one.

Most adrenalectomized animals after typhoid vaccine died within three to four hours. Despite the fact that when hormone is given 30 minutes after its injection there is a high survival rate, when given one hour after, no protection occurs.

Only 10 per cent of rats were protected when 2.0 mg. desoxycorticosterone acetate was given subcutaneously in oil solution 1 hour be-

fore 1.33 M.L.D. typhoid vaccine, while 33 per cent were protected by a similar dose in aqueous solution, given intravenously or intraperitoneally.

Normal mice require two injections of 1 ml. of typhoid vaccine at an interval of 16 hours to kill them. Adrenal extract given at the times of its injection afforded no protection or when given 32 and 24 hours before and at the times of injection of vaccine.

Adrenal extract, when given to mice infected with pneumococci type I, did not modify the fatal course of the disease (Chart 3, graph 1). However, mice infected with pneumococci type III appeared to receive minimal protection, i.e., the speed with which the infection proved fatal was decreased. Further, three of 22 extract-treated mice were alive 120 hours after inoculation, while all non-extract-treated animals were dead in 96 hours, and 96 per cent in 72 hours (Chart 3, graph 2).

DISCUSSION

The protective power of adrenal hormones against the action of bacterial toxin, whether endotoxin such as typhoid, or exotoxin as botulinus, or a combination as diphtheria toxin, seems to parallel its action on carbohydrate rather than electrolyte metabolism. For example, in the protection given adrenalectomized rats against botulinus toxin by adrenal extract, the gluconeogenic action of the hormone probably plays an important part. The food consumption of animals following botulinus toxin is greatly decreased and such partial starvation of adrenalectomized animals results in extreme hypoglycemia. Administration of "carbohydrate active" principles of the adrenal, however, maintains the blood glucose at normal levels. Whether force feeding of adrenalectomized rats during botulinus intoxication would have proved beneficial was not determined.

The synergistic action of compound A acetate and desoxycorticosterone acetate in protecting adrenalectomized rats against typhoid vaccine is interesting. It indicates the desirability of maintaining both electrolyte and carbohydrate metabolism in combatting intoxication.

Evidence indicates that adrenal extract may exert its protective effects rapidly. Death occurred in many rats within 4 hours after typhoid vaccine injection in the absence of extract. But if extract is given as long as 30 minutes after the vaccine, toxic action may be prevented.

That normal animals infected with pneumococci type III were somewhat protected by adrenal extract, while those infected with type I were not, recalls the fact that type III pneumococci are much more toxic. It is possible that the adrenal hormone combatted the toxic rather than invasive component of the infection. Conceivably, in highly toxic infections the use of adrenal extract might be helpful.

Although increased survival of normal rats treated with large doses of adrenal extract subjected to peptone shock has been reported

(Ingle, 1944), extract does not protect normal rats against diphtheria toxin (Ingle, 1947) nor normal mice against typhoid vaccine.

The fact that the lethal dose of crystalline tetanus toxin is the same for normal and adrenalectomized rats is in agreement with earlier observations (Rogoff, 1927) in which less highly purified toxin preparations were used. Grollman (1936) has questioned the completeness of adrenalectomy in the earlier investigations. In the present studies, any possibility of remnants of adrenal tissue was ruled out by testing all surviving rats after the toxin studies for completeness of adrenalectomy and not including results obtained on any animal with evidence of adrenal tissue (Lewis and Page, 1946).

SUMMARY

The M. L. D. of botulinus toxin was approximately 4.5 times greater for normal than adrenalectomized rats. Adrenal extract or compound A acetate was effective in protecting adrenalectomized rats against botulinus toxin.

The M.L.D. of diphtheria toxin was approximately 5 times greater for normal than adrenalectomized rats. Adrenal extract protected adrenalectomized rats, while compound A acetate was only partially effective.

The M.L.D. of crystalline tetanus toxin was 60 mouse M.L.D. for both normal and adrenalectomized rats. No protection was afforded adrenalectomized rats by adrenal extract or desoxycorticosterone acetate.

Adrenal extract (0.5 ml. twice daily) gave no protection to normal mice against lethal doses of typhoid vaccine.

Adrenal extract (0.2 ml. twice daily) gave no protection to normal mice infected with type I pneumococci, while a slight beneficial effect resulted when given mice infected with type III pneumococci.

ACKNOWLEDGMENTS

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REFERENCES

Lewis, L. A., and I. H. Page: *J. Lab. & Clin. Med.*, 31: 1325. 1946.
Pillemer, L., and W. B. Wartman: *Federation Proceedings.* 6: 398. 1947.
Ingle, D. J.: *Am. J. Physiol.* 142: 191. 1944.
Ingle, D. J.: *Exper. Med. & Surg.* 5: 375. 1947.
Rogoff, J. M., and E. E. Ecker, *Am. J. Physiol.* 80: 200. 1927.
Grollman, A.: The Adrenals. Williams and Wilkins Co., Baltimore, Md., p. 267, 1936.

GROWTH INHIBITION IN BONE AND BONE MARROW FOLLOWING TREATMENT WITH ADRENOCORTICOTROPIN (ACTH)

BURTON L. BAKER AND DWIGHT J. INGLE

From the Department of Anatomy, University of Michigan Medical School and Research Laboratories, The Upjohn Company

ANN ARBOR AND KALAMAZOO, MICHIGAN

THE ACTION of ACTH on the tibia of immature rats has been studied by Becks, Simpson, Li and Evans (1944) and by Becks, Simpson, Marx, Li and Evans (1944). These workers observed retardation of chondrogenesis and osteogenesis to follow treatment of immature rats with ACTH. Likewise, ACTH antagonized the action of growth hormone when both were injected into hypophysectomized rats. The experiments now being reported supplement these observations by adding information regarding the action of ACTH on the bones of (1) adult rats, (2) under varied dietary conditions controlled by tube feeding and (3) at higher dose levels. In addition, the effect of ACTH on the bone marrow and vertebrae will be discussed.

MATERIALS AND METHODS

The general plan of attack for these experiments has been outlined in detail elsewhere (Baker, Ingle, Li and Evans, 1948). Adult male rats (Sprague-Dawley strain) were force-fed diets high in carbohydrate, fat or protein. Information is given in table 1 concerning the doses of ACTH injected, the duration of the experiments and the number of animals used. The ACTH was prepared by the method of Li, Evans and Simpson (1943) and each daily dose was divided and given in 8 injections. The controls received 0.9% NaCl. At autopsy, the proximal ends of the tibia and 2 to 3 vertebrae were removed for study, decalcified in 5% trichloracetic acid, sectioned sagitally and stained with hematoxylin and eosin and the Masson procedure. The width of the epiphyseal cartilage was determined microscopically by taking the average of three measurements, one from the center of the cartilage and one each near the anterior and posterior extremities.

OBSERVATIONS

Tibia

The administration of ACTH to adult male rats caused histological modification of the epiphyseal cartilage, the zone of bone formation and of the bone marrow. Since the duration of the experiment proved to be an important factor in the induction of these changes, the effects of treatment over the longest period of time and at the highest dosage (3 mg. per day for 21 days) will be described first.

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Epiphyseal cartilage. As is apparent from table 1, measurement of the epiphyseal cartilage showed it to be considerably more marrow in the experimental than in the control rats. This reduction in width resulted from a number of intrinsic changes. First, inhibition of cartilage cell proliferation was indicated by the significant reduction in the number of cartilage cells in the epiphyseal plates. Additional evidence of this suppression was furnished by degenerative changes which were observed in the cells of the proliferation zone (figs. 1 and 2). A majority of these cells were atrophic and possessed pycnotic nuclei. In addition, the cell columns in some cases were somewhat more irregularly arranged. This atrophy of the epiphyseal cartilage was reflected also in the zone of cell and lacunar enlargement. Here, as compared with the controls, there was definite interference with the enlargement of the cells and fewer of them contained intra-cellular vacuoles. Also, the lacunae failed to enlarge as extensively as in the controls. In the treated rats, the invasion of the enlarged lacunae by medullary blood vessels was impaired significantly.

Second, the epiphyseal cartilage was thinner after treatment with ACTH because of reduction in the amount of matrix which it contained. This change appeared to have resulted from the impaired capacity of the atrophic cartilage cells to produce it. In some of the treated rats the matrix, except for the lacunar capsules, stained less intensely with aniline blue suggesting that the chemical or physical character of the matrix had been modified. This was a rather variable observation.

Zone of bone formation. In the control rats, numerous large, active osteoblasts were present in the area of bone formation near the epiphyseal cartilage (fig. 1). In contrast, after treatment with ACTH bone formation was suppressed as shown by a profound and general atrophy of these osteoblasts (fig. 2). It was more difficult to ascertain whether or not the osteoclasts were affected also. They were still present after treatment with ACTH but in some cases may have been reduced slightly in number. They were not affected as noticeably as the osteoblasts.

The impaired osteogenesis manifested itself in great variation in the pattern of the metaphyseal trabeculae. In the controls, most of the inter-columnar septa of cartilaginous matrix projected into the metaphysis as calcified spicules. Frequently, they extended for a short distance without a covering of newly-formed bone. Treatment with ACTH induced variable changes in this osteogenetic region. In some of the rats at the 3 mg. dosage, many of the inter-columnar septa did not project as spicules, leaving considerable areas of the osteogenetic zone free of bone trabeculae. This suggested that in these cases resorption of the labile metaphyseal trabeculae was proceeding more rapidly than their construction. However, in all of the other cases treated at this dose level, the bony trabeculae were broader, "gnarled" in ap-

pearance and less regularly arranged than in the control animals (fig. 2). Likewise, osseous tissue tended to cover completely all of the cartilaginous spicules even extending up on to the epiphyseal plate itself (fig. 2). This was suggestive of the beginning of a "sealing off" of the cartilage by bone which has been observed to occur after hypophysectomy in the rat. The line of junction of the epiphyseal cartilage with the zone of bone formation was quite irregular. This probably resulted from a combination of impaired invasion of the cartilage lacunae and retarded osteogenesis. A significant widening of the marrow sinusoids was not observed in contrast to observations made in immature rat (Becks, Simpson, Li and Evans, 1944).

Detectable modification of the shaft or bony portion of the epiphysis did not occur.

Bone marrow. At the 3 mg. dosage level, ACTH caused a consistent atrophy of the bone marrow which was particularly striking in the center of the shaft (figs. 3 and 4) where normally the red marrow is most dense. This atrophy also involved the marrow of the region of bone formation and, usually the epiphysis as well. The character of this change resembled somewhat "serous atrophy" which has been observed in the bone marrow under various experimental conditions such as under-feeding. In the usual histological preparations, it appeared that fat had increased at the 3 mg. dosage level (3 experimental and 3 control rats) and an attempt was made to verify this observation by staining frozen sections of the bone marrow for fat. The observations made on these preparations of the relative amounts of fat in the marrow of the control and experimental rats were inconclusive. However, it should be pointed out that we did not take precautions to select sections from comparable regions of the bone marrow. These preparations for fat did show that the large spaces of figures 3 and 4 contained fat and, therefore, it seems safe to infer that fat was increased in the bone marrow of the tibia after injection of ACTH. Many megakaryocytes remained in spite of the extensive atrophy of the marrow and did not seem to have undergone much of a decline in number.

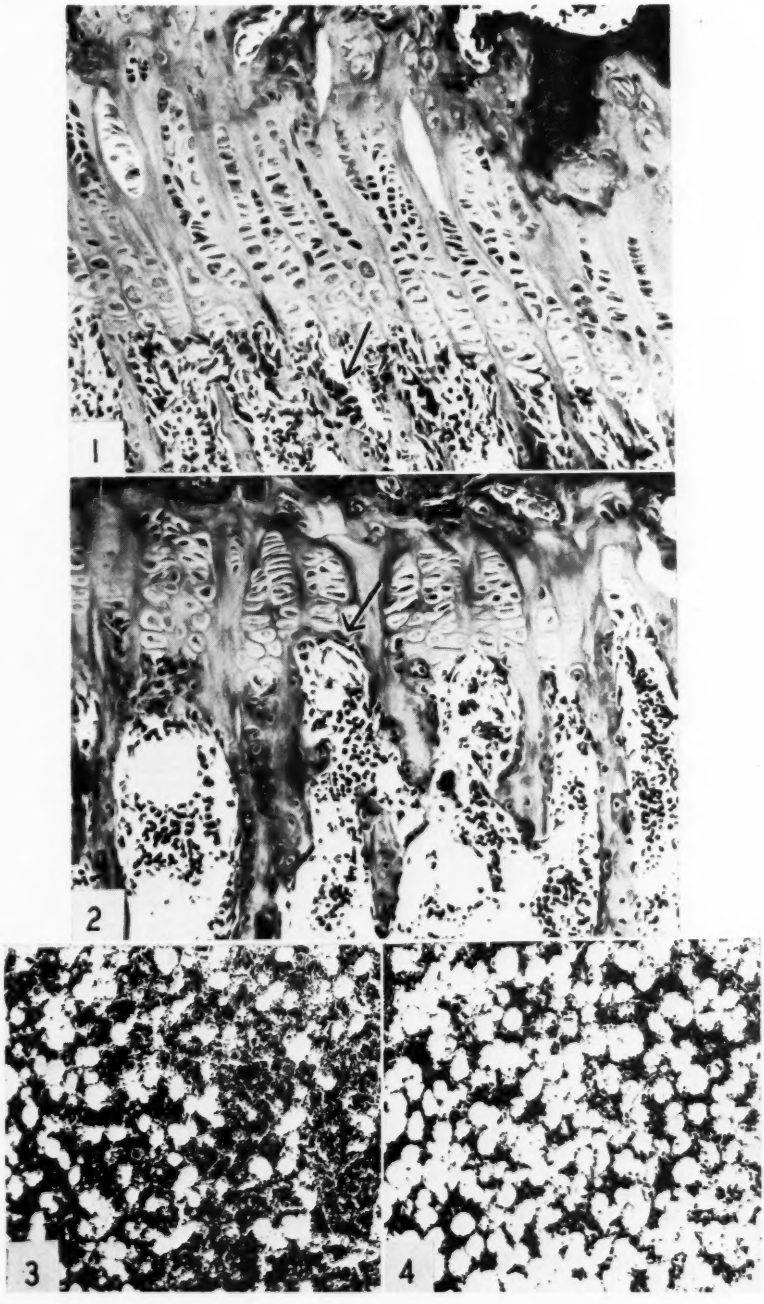
Insofar as could be ascertained by histological study, modification of the diet did not affect consistently the structural changes which followed injections of ACTH.

FIG. 1. Rat 901. Control, high carbohydrate diet. Epiphyseal cartilage of tibia. At the end of the pointer are several active osteoblasts. Spicules of cartilage, on which bone has not yet been laid down, extend into the osteogenetic zone. Masson. ×184.

FIG. 2. Rat 1001. One mg. ACTH daily for 21 days, high carbohydrate diet. Cartilage is thinner than in figure 1. There are fewer cells in the columns and those of the proliferating zone are atrophic. The cells of the resorption zone are few in number and smaller. None of the smaller lacunae are being invaded by capillaries. Osteoblasts are atrophic and some bone (arrow) caps the epiphyseal cartilage. Masson. ×184.

FIG. 3. Rat 18003. Control, medium carbohydrate diet.
Bone marrow of shaft. Masson. ×75.

FIG. 4. Rat 17003. Three mg. of ACTH daily for 21 days, medium carbohydrate diet. The red marrow is atrophic and seems to be replaced by fat. Masson. ×75.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On the 1 mg. dosage for 21 days the effects on the bones and bone marrow were essentially similar to those described for the 3 mg. dosage level. The atrophy of the red marrow and the inhibition of chondrogenesis and osteogenesis were equally as marked at this lower dose. Although the one ACTH-treated, protein-fed animal did not exhibit a reduction in width of the epiphyseal cartilage, nevertheless, all of the other histological changes which have been shown to follow such hormone treatment were present.

In general, treatment with 8 mg. of ACTH per day over the shorter period of 10 days did not affect the bone as much as did treatment at

TABLE 1. EFFECT OF VARIED DOSES OF ACTH ON WIDTH OF THE EPIPHYSEAL CARTILAGE

Diet	1 mg. daily for 21 days		3 mg. daily for 21 days		8 mg. daily for 10 days	
	No. Rats	Mean width (μ)	No. Rats	Mean width (μ)	No. Rats	Mean width (μ)
Carbohydrate						
Experimental	2	102	5[1]	118	4	129.3
Control	2	218	4[1]	165	2	166
Fat						
Experimental	2	110	2	97	2	139
Control	2	142	1	148	2	147
Protein						
Experimental	1	162	2	97	2	132
Control	1	154	1	148	1	159

[1] Three experimentals and 3 controls were fed a medium instead of high carbohydrate diet.

the lower doses for 21 days. As shown by table 1, it was possible to demonstrate a narrowing of the epiphyseal cartilage by direct measurement. However, significant histological modifications were not readily evident upon microscopic examination. Thus, the effect on chondrogenesis and cartilage resorption was not very great. Impairment of osteogenesis had occurred as shown by a definite reduction in the number of osteoblasts. Likewise, the "gnarling" and distortion of the trabeculae appeared under this dosage, in most cases with bone having been deposited on the cartilaginous spicules up to the epiphyseal plate itself. Atrophy of the red marrow occurred although not as extensively as after the longer period of treatment.

Vertebra

The bodies of the vertebrae were studied in the rats treated for 21 days with a daily dose of 1 or 3 mg. of ACTH. In contrast to the tibia, it was far more difficult to detect histological changes. No consistent modification was found in the cartilage at the superior and inferior ends of the vertebrae, in density of the trabeculae or in thickness of the shaft. The bone marrow showed a slight atrophy of the red marrow after ACTH with some reduction in the density of the constituent

cells usually being evident. However, these effects did not approximate in intensity those which occurred in the tibia.

DISCUSSION

The retardation of chondrogenesis and osteogenesis assumes its greatest significance when viewed in the light of the growth-inhibiting potentiality of ACTH. Apparently, these end-effects are elicited through the stimulated release by the adrenal cortex of the 11-oxysteroids. The literature supporting the concept of growth inhibition by ACTH and the 11-oxysteroids is considerable and has been reviewed elsewhere (Baker and Whitaker, in press). It has been shown that numerous proliferating tissues show evidence of atrophy after treatment with ACTH. Among these might be listed the lymphoid tissue (Dougherty and White, 1945), epiphyseal cartilage (Becks Simpson, Li and Evans, 1944), hair and epidermis (Baker, Ingle, Li and Evans, in press).

A negative nitrogen balance was induced in these rats by ACTH (Ingle, Prestrud, Li and Evans, 1947). Presumably, at least part of this loss of nitrogen was the end-result of acceleration of the process of gluconeogenesis in the liver. To what extent tissues other than the liver contribute to this loss of nitrogen is not known at the present time. Also, it is not clear whether this loss of nitrogen is a cause or an effect of the growth inhibition induced by ACTH. The local inhibition of hair and epidermal growth by one of the 11-oxysteroids (11-dehydro 17-hydroxycorticosterone) shows that the liver is not necessarily a link in the chain of events leading to growth inhibition (Whitaker and Baker, in press).

The bone marrow changes should be considered from 2 view-points: the effect of ACTH on (1) protein metabolism and growth and (2) on fat metabolism. It is probable that the atrophy of the red marrow fits into the pattern of growth inhibition of proliferating tissues brought about by ACTH and, thus, the probable augmentation of fat in the marrow may be secondary to the reduction in volume of the red marrow. On the other hand, a considerable body of evidence shows that the adrenal cortex may be involved more directly in lipid metabolism, especially in its transport (Ingle, 1943). In fact, fatty infiltration of the liver of rats treated with ACTH and fed high carbohydrate diets has been demonstrated previously (Baker, Ingle, Li and Evans, 1948).

The marked atrophy of the red marrow of the tibia after treatment with ACTH suggests the possibility that either an anemia or a granulocytopenia might exist in these animals. Such blood studies have not been reported in the rat but in the mouse White and Dougherty (1945) found ACTH to elicit an increase in the number of red cells and polymorphonuclear leucocytes. These seemingly contradictory findings are difficult to evaluate at this time. It is to be noted that all bone marrow may not share in the atrophy which occurs in the tibia.

At least, the marrow of the vertebrae showed little change.

To a variable degree the changes which we have found to be elicited by treatment of rats with ACTH resemble those which follow underfeeding of rats (Saxton and Silberberg, 1947) and the feeding of diets deficient in various factors of the vitamin B complex to mice (Levy and M. Silberberg, 1946; Levy and R. Silberberg, 1946; Nelson, Sulon, Becks and Evans, 1947; Silberberg and Levy, 1948; Silberberg, Levy and Younger, 1948). This similarity raises the question of the nutritional condition of our animals. Food intake was controlled by tube-feeding. The possibility that ACTH may impair absorption through the intestinal wall has not been examined. Likewise, such similarities of response under these varied experimental conditions suggest an inter-relation between the action of vitamins and ACTH on the peripheral tissues.

The findings in this study are of interest in relationship to the experimental reproduction of the symptoms of Cushing's disease. Especially is this true in view of the position of Albright (1947, p. 329): " . . . I think it is safe to conclude that whatever bone changes are found in Cushing's Syndrome are the result of an excess production of 'S' hormone." By "S" hormone Albright refers to at least some of the adrenal cortical steroids which we have designated as the 11-oxysteroids (gluconeogenic) and which, on the basis of physiological grounds (Ingle, Prestrud, Li and Evans, 1947) and the similarity of the effects of ACTH (Baker, Ingle, Li and Evans, submitted for publication) and 11-oxysteroids (Baker and Whitaker, in press) on the skin, appear to be the substances which are secreted at an accelerated rate when the adrenal cortex is stimulated by Li's ACTH.

Many of the symptoms of Cushing's syndrome have been reproduced experimentally by the administration of ACTH or adrenal cortical steroids. Of interest here is the possible appearance of osteoporosis. As pointed out by Albright (1947, p. 296): "In osteoporosis the decrease of bony tissue is due to the fact that the osteoblasts lay down too little bony matrix; that matrix which is laid down is normally calcified." Over the period of our experiment we did not induce osteoporosis. The essential condition for its appearance, however, had been elicited, namely, a suppression of osteoblastic activity. It seems entirely possible that if the suppression of bone formation were maintained by prolongation of treatment with ACTH that the continued resorption of bone to meet the usual bodily demands ultimately might lead to a state of osteoporosis. This outcome would be dependent on at least a normal rate of resorption. The extent to which resorption is affected by ACTH was not ascertained from our studies. The recent finding of Bartter, Forbes and Albright (1948) that treatment of human cases of panhypopituitarism, ovarian agenesis and osteitis deformans with ACTH may cause an increased excretion of urinary calcium is pertinent in this connection. They explained this effect on the basis of continued destruction of bone in the face of failure of os-

teoblasts to form bone matrix when under the influence of adrenocorticotropin.

The concept outlined above holds that the primary effect of ACTH on bone is suppression of bone formation rather than increased withdrawal of bone. In respect to bone, this concept agrees with the hypothesis of Albright (1947) which postulates that the action of the "S" hormones is an "anti-anabolic" one rather than "catabolic." Thus, it appears probable from a study of the effect of ACTH on bone that in the peripheral tissues the 11-oxysteroids inhibit the synthesis of protein rather than accelerate its break-down.

In the vertebrae, where in human Cushing's disease osteoporosis is more likely to occur, we did not find evidence of osteoporosis and only an insignificant difference in osteoblastic activity between the experimentals and controls. These findings do not preclude the possibility that osteoporosis might be reproducible in the rat with ACTH. There is evidence that a refractory state develops during repeated injections of this hormone (Anderson, Page and Li, 1947). Thus, with continued treatment the rats may have been losing their capacity to respond to the action of ACTH.

SUMMARY

The following changes were observed in the tibias of adult male rats treated with ACTH in doses of 1 and 3 mg. daily for 21 days: (1) retardation of chondrogenesis and osteogenesis and (2) atrophy of the red marrow with apparent replacement of it with fat. Variation of the diet which was controlled by tube-feeding did not modify these results significantly. Less noticeable changes were observed in the vertebrae.

REFERENCES

Anderson, E., E. W. Page and C. H. Li: *Endocrinology* 41: 105. 1947.
Albright, F.: *Recent Progress in Hormone Research* Vol. 1, p. 293. 1947. Academic Press Inc., New York.
Baker, B. L., D. J. Ingle, C. H. Li and H. M. Evans: *Am. J. Anat.* 82: 75. 1948.
Bartter, F. C., A. P. Forbes and F. Albright: *Proc. Amer. Assoc. for the Study of Internal Secretions*, 1948.
Becks, H., M. E. Simpson, C. H. Li and H. M. Evans: *Endocrinology* 34: 305. 1944.
Becks, H., M. E. Simpson, W. Marx, C. H. Li and H. M. Evans: *Endocrinology* 34: 311, 1944.
Dougherty, T. F., and A. White: *Am. J. Anat.* 77: 81. 1945.
Ingle, D. J.: *J. Clin. Endocrinol.* 3: 603. 1943.
Ingle, D. J., M. C. Prestrud, C. H. Li and H. M. Evans: *Endocrinology* 41: 170. 1947.
Levy, B. M., and M. Silberberg: *Proc. Soc. Exper. Biol. & Med.* 63: 380. 1946.
Levy, B. M., and R. Silberberg: *Proc. Soc. Exper. Biol. & Med.* 63: 355. 1946.
Li, C. H., H. M. Evans and M. E. Simpson: *J. Biol. Chem.* 149: 413. 1943.
Nelson, M. M., E. Sulon, H. Becks and H. M. Evans: *Proc. Soc. Exper. Biol. & Med.* 66: 631. 1947.
Saxton, J. A., and M. Silberberg: *Am. J. Anat.* 81: 445. 1947.
Silberberg, M., B. M. Levy and F. Younger: *Proc. Soc. Exper. Biol. & Med.* 67: 185. 1948.
Silberberg, R., and B. M. Levy: *Proc. Soc. Exper. Biol. & Med.* 67: 259. 1948.
White, A., and T. F. Dougherty: *Endocrinology* 36: 16. 1945.

INFLUENCE OF QUANTITATIVE THYROPROTEIN TREATMENT OF HENS ON LENGTH OF INCUBATION PERIOD AND THYROID SIZE OF CHICKS[1]

ROBERT S. WHEELER AND EDMUND HOFFMANN

From the Department of Poultry Husbandry

THE UNIVERSITY OF GEORGIA, ATHENS, GEORGIA

PREVIOUS WORK in this laboratory has shown that chicks hatched from eggs laid by thyroprotein-fed hens have greatly enlarged thyroids (Wheeler and Hoffman, 1948a). The presence of goiters suggested that the chicks might be hypothyroid and therefore, might exhibit reduced rate of embryonic development. A preliminary study of this aspect of the problem established that eggs of hens fed thyroprotein at a dietary level of 0.02% required 6 to 12 hours longer to hatch (Wheeler and Hoffman, 1948b). Both of these findings have recently been confirmed by the independent studies of McCartney and Shaffner (1948).

The present study was designed to determine the interrelationships between degree of goiterogenicity, increase in length of incubation period, and level of thyroprotein fed with the view of attempting to explain the mechanisms involved.

MATERIALS AND METHODS

To secure eggs for hatching, 60 year-old New Hampshire hens were divided at random into four groups of 15 birds. The hens were kept on litter-covered floors in adjacent pens within the same house. A single male of the same stock was rotated daily among the pens in order to minimize genetic variability of the chicks. One setting of eggs, made during a pre-treatment period established that chick thyroid weight and mean incubation time were comparable among groups. Synthetic thyroprotein[2] was then added to the diet of three of the groups of hens at levels of 0.02%, 0.04%, and 0.08% respectively, while a fourth group was continued on the control diet. The formula of the basal diet has been published previously (Hoffmann and Wheeler, 1948). Eggs were set for hatching after the hens had been on this regime for an average of 14, 21, and 28 days.[3] Treatment was then discon-

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[2] Synthetic thyroprotein (iodinated casein) was generously supplied by Dr. W. R. Graham, Jr., Cerophyl Laboratories, Kansas City, Mo. This product contains 3.0% thyroxine according to the manufacturer's chemical assay.

[3] Eggs were collected during a period of at least seven days before setting. Eggs representing an average of 14 days on treatment were laid from the 10th through the 17th days, (10+17)/2=14; 21 days=18–24; 28 days=25–31.

tinued and all groups were fed the control diet. Eggs were set again when the hens had been off the experimental diets for an average of 4, 12, 19, and 29 days,[4] in order to determine the residual effects of treatment.

Length of incubation period was determined by counting the number of chicks appearing in two-hour intervals during the hatch. A total of 866 chicks was involved. Mean incubation time was calculated and evaluated by analysis of variance for subsamples with different numbers of individuals (Snedecor, 1946).

On the day following hatching, a sample of chicks from each group was sacrificed by decapitation, the thyroids were quickly removed and weighed to 0.1 mg. on a Roller-Smith balance. Each sample contained five males and five females.[5]

Since it is well known that seasonal factors influence rate of thyroidal activity it is important to note that these observations were made from February through May 1948.

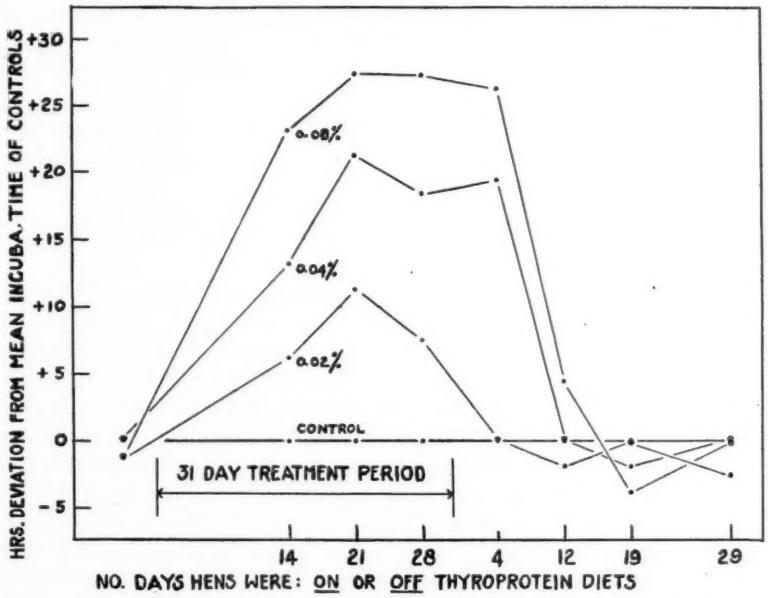

FIG. 1. Delayed Hatching of Chicks Following Administration of Quantitative Levels of Synthetic Thyroprotein to their Dams.

RESULTS

Delayed hatching effect. Length of incubation time was almost proportional to the amount of thyroprotein administered to the hens.

[4] An average of 4 days = 0–8; 12 days = 9–15; 19 days = 16–22; 28 days = 23–32.

[5] Day-old female chicks consistently show slightly larger thyroids than male chicks. When small numbers of chicks are compared it is difficult to show statistical significance of this difference. Aberle and Landauer (1935) were able to show a significant sex difference with more than 900 weights. Our unpublished data confirm these findings.

Mean incubation time was increased 6–11, 13–21, and 23–37 hours respectively when thyroprotein was fed at levels of 0.02%, 0.04%, and 0.08% of the diet (Figure 1, and Table 1). The ratio of response was 1:2:3, whereas the ratio of treatment was 1:2:4.

It will be noted (Figure 1) that the maximal effect occured almost at once and that there was no cumulative increase in incubation time after an average of 14 days on the experimental diets.

Statistical analysis of the data shows that the differences between each level, as well as between treatments and control are highly significant except in three instances in which significance lies at the 5.0% level.

TABLE 1. MEAN TIME REQUIREMENT FOR INCUBATION OF EGGS COLLECTED BEFORE, DURING, AND AFTER THYROPROTEIN TREATMENT OF HENS

Level thyroprotein in diet of hens	Mean length of incubation period (Hrs.)[1]							
	Before treatment	Ave. no. days hens were on exptl. diets			Ave. no. days hens were off experimental diets			
		14	21	28	4	12	19	29
None	519	517	518	519	518	515	518	516
0.02%	518	523	530	526	518	513	518	514
0.04%	519	540	540	537	537	516	517	516
0.08%	518	550	546	546	544	519	515	516

[1] All values for 14, 21, 28 days *on*, and for 4 days *off* the experimental diets are highly significantly different from each other except as noted in text.

The delayed-hatching effect had disappeared within an average of 12 days after treatment was discontinued. There was, however, evidence of residual effect. After an average of 4 days off treatment mean hatching time was normal for the 0.02% lot but the delay persisted at the 0.04% and 0.08% levels. The latter values are highly significantly different from the control value; however, the difference between 0.04% and 0.08% diets is significant only at the 5.0% level.

Other data on the delayed hatching effect involving large numbers of chicks (160 controls and 400 experimentals in two hatches) from hens fed thyroprotein at the 0.02% level, have been previously presented (Wheeler and Hoffman, 1948b). Examination of these data shows the delayed hatch follows a normal curve which is somewhat platykurtic as compared to the curve for the control chicks.

Goiterogenic effect. Although the thyroids of chicks from hens on the experimental diets were, in every case, highly significantly heavier than those of the control chicks, the degree of goiterogenicity was not directly proportional to the amount of thyroprotein in the hen's diet (Table 2, Figure 2).

Differences between the 0.02% level and either the 0.04% and 0.08% levels are highly significant for all hatches of eggs from treated hens as well as for the hatch of eggs collected an average of four days

TABLE 2. MEAN THYROID WEIGHT OF TEN-CHICK SAMPLES (5 MALES, 5 FEMALES) BEFORE, DURING, AND AFTER TREATMENT OF THEIR DAMS WITH THYROPROTEIN

Level thyroprotein in diet of hens	Mean thyroid weight (mgs.) of 2-day-old-chicks							
	Before treatment	Av. no. days hens were on exptl. diets			Ave. no. days hens were off experimental diets			
		14	21	28	4	12	19	29
None	4.92	3.87	3.66	3.62	3.43	5.00	5.25	4.89
0.02%	4.90	5.28†	6.30†	5.45†	5.98†	5.25	3.90*	4.35
0.04%	5.14	8.50†	8.68†	8.02†	8.97†	6.55	4.20	4.29
0.08%	5.19	8.35†	7.84†	7.23†	6.99†	4.99	3.75*	4.25

† Highly significantly heavier than corresponding control thyroids (1.0% level).
* Significantly lighter than corresponding control thyroids (5.0% level).

after the control diets were resumed. The difference between the 0.04% and the 0.08% level is statistically insignificant in all cases except one, four days after treatment ceased, when the thyroid weights of chicks from the 0.08% level hens were significantly smaller. Moreover, hens fed the 0.04% level consistently produced chicks with the heaviest thyroids. Next in descending order of thyroid weight were the chicks of hens on 0.08%, 0.02% and control diet (Figure 2). Thus it is apparent the relationship between level of thyroprotein fed

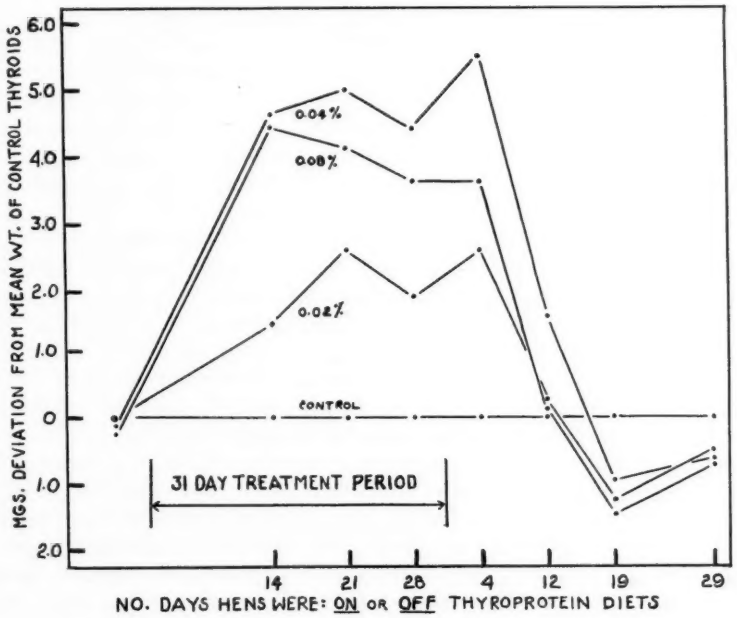

FIG. 2. Goiterogenic Effect in Chicks from Eggs Laid by Hens Fed Quantitative Levels of Synthetic Thyroprotein.

and the degree of goiterogenicity is not proportional beyond the 0.04% level. No greater response was obtained with the 0.08% than with the 0.04% level.

Thyroid enlargement was maximal 14 days after the hens were put on the experimental diets and there were no further increases when treatment was continued for 21 or 28 days. Thyroid weight was not decreased in chicks hatched from eggs laid four days after the experimental diets were discontinued. (This is in contrast to the observations on mean length of incubation period, since by this time the effect of thyroprotein had completely disappeared in chicks from hens fed the 0.02% level.) By the time the hens had been off treatment for 12 days, thyroid weights of their chicks fell within the normal range. However, at the next hatching date (19 days off treatment) there was a consistent sub-normal depression of thyroid weight in chicks from hens that had been treated. The decrease is statistically significant for the 0.02% and 0.08% levels of treatment.

Figure 1 reveals that increase in thyroid weight reaches a maximum level shortly after treatment is initiated and following cessation of treatment there is a residual effect during the first week. This is followed by a sharp depression in thyroid weight below the normal range. Twenty-nine days subsequent to the end of treatment an apparent return to normal thyroid weight was observed.

DISCUSSION

The present experimental results show that hens fed supplemental thyroxine in the form of synthetic thyroprotein produce offspring which: (1) require a longer incubation period, and (2) exhibit marked thyroid enlargement. These two phenomena occur concomitantly and appear to be rather directly associated with each other and with the dosage used except in the instances noted.

One of the instances concerns the response to quantitative dosage. Delayed hatching response appears to be almost directly related to dosage level whereas thyroid enlargement is proportional only for the two lower dosages. The lack of additional goiterogenicity with levels of thyroprotein in excess of 0.04% may be due, however, to inability of the chick thyroid to respond further. Bates, Riddle and Lahr (1941) have demonstrated the existence of thresholds of response of the (newly-hatched) chick thyroid to injected thyrotropin as well as a genetic difference between strains in ability to respond to progressively larger dosages. The work of McCartney and Shaffner (1948) tends to support our view that the maximum goiterogenic response of the chick may have been reached. They obtained chick thyroids 1.5 and 2.75 times heavier than the controls by feeding thiouracil to hens at levels of 0.1% and 0.3% respectively. These values are comparable to the ones obtained in the present study with 0.02% and 0.04% thyroprotein. It may be reasoned that, since thiouracil is an extremely

potent goiterogenic agent, and since they found the higher level approached the maximal level of tolerance of this drug, the goiters produced in the present study were as large as could be produced.

The other exception concerns the time of appearance and of disappearance of the two effects. At the low level of treatment the delayed hatching effect appeared first after treatment started and was less persistent when treatment ceased, than was the goiterogenic effect. However, the absence of these "time" effects at either of the two high dosage levels suggests the differences are of minor significance.

The present experimental data indicate a definite "transfer effect" from hens to their chicks via the egg. Riddle (1930) first advanced the suggestion that maternal thyroid hormone deposited in the egg plays a role in regulating rate of embryonic development and length of incubation period. Riddle (1930) and Hollander and Riddle (1946) observed that pigeons with naturally occuring goiters and symptoms suggestive of myxedema required an extra day or two to hatch. Correction of the dams' hypothyroidism with supplemental iodine corrected both the goiter and delayed hatching of the offspring. Landauer (1942) described a "maternal effect" in which Frizzle mothers apparently failed to deposit in their eggs all the substances essential for normal development. In a previous paper, Aberle and Landauer (1935) showed that day-old homozygous Frizzle chicks had larger thyroids than did the White Leghorns used as controls. Ukita (1919) reported prolonged gestation and thyroid hypertrophy in the young of rabbits thyroidectomized during pregnancy. In all these cases of maternal "transfer effects," the dams were hypothyroid and produced young with enlarged thyroids which seems to substantiate Riddle's hypothesis that maternal thyroid hormone is transferred to the developing embryo. There is a striking similarity between these cases and the data reported here.

Although the mechanisms involved in the production of goiterous offspring which require an increased incubation period are not known, it is possible to postulate at least three explanations. These effects could be due to: (1) reduced deposition of maternal thyroid hormone in the egg; (2) direct transfer of thyroprotein substance(s); (3) a combination of these two factors.

Perhaps the most logical way of explaining the results presented in this paper is to assume that maternal thyroid hormone deposited in the egg influences the size and function of the chick thyroid and length of incubation period. Then, insufficient maternal hormone in the egg might be expected to be reflected in an increased secretion of thyrotropin by the chick pituitary thus causing enlargement of the chick thyroid. The delayed hatching could result from reduced rate of embryonic development or the ineffective functioning of the chick thyroid. Conversely an oversupply of maternal hormone in the egg

might result in thyroid reduction. This is merely an extension of the Moore-Price phenomenon in which excessive maternal thyroid hormone in the egg would inhibit the thyrotropic activity of the chick pituitary and a deficiency of maternal hormone would have the opposite effect.

This postulation is supported by ample evidence that both the thyroid and pituitary of the developing chick are functional prior to hatching. The chick thyroid produces colloid beginning on the 10th day of incubation and in increasing amounts thereafter (Hopkins, 1935). In addition, iodine determinations also indicate increasing thyroid activity from the 10th day to hatching (Sun, 1933). Moreover, amphibian larvae tests indicate the presence of active thyroid hormone in the gland beginning on the 10th day and in quantities on the 18th day (Hopkins, 1935; Uhlenhuth and Ebeling, 1925). Fugo (1940) has shown that the chick thyroid will differentiate in the absence of the pituitary but its functional activity and growth subsequent to the 14th day of incubation are dependent upon hypophyseal stimulation. He did not report that any of his hypophysectomized chicks hatched although one chick lived within the shell for eight days after the control chicks had hatched. It is thus likely that the presence of a functional thyroid controls the mechanism and the time of hatching.

In order to reconcile the present data with the proposed hypothesis it is necessary to assume that the thyroidal substance (thyroxine) in synthetic thyroprotein is not deposited within the egg in significant quantities. Turner, Irwin, and Reineke (1945) were unable to detect the presence of thryoidal (or antithyroid) substance in the eggs of thyroprotein-fed hens, and McCartney and Shaffner (1948) were unable to modify chick thyroid weight by injecting crystalline d,l-thyroxine into normal hens. Examination of these data suggests that the thyroactive substance in synthetic thyroprotein differs biologically from natural thyroprotein (thyroid hormone) in not being deposited in the hen's egg.

It is likely that the presence of thyroid hormone or of an effectively functioning chick thyroid may be necessary for the process of hatching (Fugo, 1940). Furthermore, deviations in amount of thyroid hormone production or presence at or near hatching appear to determine whether hatching will be delayed. For instance, Grossowicz (1946) was able to delay hatching proportionately to the amount of thiourea he injected into normal eggs. With the largest dosage the chicks hatched 9 to 10 days late. To return to the present data the tendency toward slightly accelerated hatching and concomitant decrease in chick thyroid weight following the cessation of treatment may be explained as the result of greatly increased maternal thyroid activity. This would be expected to follow the removal of the inhibiting influence of thyroprotein on the hen's pituitary. The data suggest

that removal of inhibition is followed by temporary overproduction of maternal thyroid hormone and a subsequent return to normal thyroid activity. Similar overstimulation of a target organ followed by a gradual return to normal has been noted in this laboratory when pellets of diethylstilbestrol have been implanted subcutaneously in young cockerels. Shortly after the pellet is completely absorbed, the testes increase markedly in size then regress to normal dimensions. There is other evidence in the literature that an oversupply of thyroidal substance in the egg can produce in the chick thyroid the opposite effect of a deficient supply. McCartney and Shaffner (1948) injected d,1-thyroxine into eggs of normal hens and thereby reduced chick thyroid size significantly.

The second hypothesis which cannot be dismissed completely at this time explains the delayed hatching and the goiterogenic phenomena as direct effects of a thyroprotein "factor" transferred to the chick through the egg. The main evidence which might support this view is the recent finding that thyroprotein fed in small quantities (0.002% of the diet) to growing chicks enhances the goiterogenic effect of thiouracil (Moreng and Shaffner, 1948). The hypothesis is untenable at the present time, however, because of the inability to detect the presence of thyroidal (or antithyroid) substances in the eggs of thyroprotein-fed hens (Turner, Irwin, and Reineke, 1945). Moreover, if these effects were due to a deposited thyroprotein factor, neither the tendency toward accelerated hatching nor decreased thyroid size (below normal) would be expected to follow cessation of treatment of the hens. The second hypothesis could not account for these "over-compensations" because the assumption of a direct goiterogenic effect of deposited thyroprotein requires a return to normal activity of the embryonic chick thyroid when the hens are replaced on the basal diet.

By means of a third hypothesis the effects of delayed hatch might be attributed to a deposited thyroprotein, or some substance associated with thyroprotein, and the goiterogenic effect to a deficient maternal thyroid hormone. The evidence upon which this postulation is based concerns the discrepancy between goiterogenicity and delayed hatching at the highest dosage level, and the "time" effects of appearance and disappearance of the two effects at the low level of treatment. However, as has been noted previously, these apparent discrepancies may be accounted for within the first hypothesis by assuming: (1) the chick thyroid is unable to respond further to thyroprotein dosages in excess of 0.04%, (2) the "time" effects to be of minor importance since they do not occur at either of the two higher treatment levels. Therefore, the first hypothesis appears to be the most tenable of the three suggested.

The first hypothesis presumes that increase in thyroid size is the result of uninhibited activity of the chick pituitary. The resulting

secretory products of the stimulated chick thyroid are apparently stored or are ineffective in inhibiting the pituitary. Storage is indicated by the presence in the goiterous thyroids of greatly enlarged, colloid-filled follicles associated with an epithelium of low height (Wheeler and Hoffmann, 1948c). Evidence that the secretory products are relatively ineffective is inferred from the fact that the chicks have lowered oxygen requirements at hatching as tested by the closed-vessel technic (McCartney and Shaffner, 1948). Thus it appears that the chicks with goiters are hypothyroid.

Whether the delayed hatching is a result of the hypothyroidism is not clear at this time. Delayed hatching could be due to: (1) reduced rate of embryonic development as a direct result of deficient maternal hormone, and/or (2) ineffective functioning of the chick thyroid during the last half of the incubation period. Further study is indicated in order to elucidate these points. It should be mentioned however, that preliminary study in this laboratory tends to indicate that rate of early embryonic development (determined by somite counts at 36 hours) is not reduced.

SUMMARY

Offspring of hens fed supplemental thyroxine in the form of synthetic thyroprotein at levels of 0.02%, 0.04%, and 0.08% of the diet require highly significantly longer periods of incubation and exhibit marked thyroid enlargement. The two phenomena occur concomitantly and appear to be rather directly associated with each other and with the dosage employed.

An hypothesis to explain these results and which best fits the present data assumes that maternal thyroid hormone deposited in the egg influences the size and function of the chick thyroid. The results appear to be due to a reduction of maternal thyroid activity and a failure of the thyroidal substance in thyroprotein to be deposited in the egg. The inhibition of the hen's thyroid results in the deposition of insufficient amounts of maternal hormone in the egg which is reflected in enlargement of the chick thyroid and delayed hatching. Release of inhibition of the hen's thyroid when the hen is replaced on the control diet is followed by deposition of an oversupply of maternal hormone in the egg which is reflected in a reduction of the chick thyroid to subnormal size and by a tendency toward accelerated hatching. Evidence is presented which suggests that the secretory products formed by the enlarged chick thyroid as a result of uninhibited pituitary stimulation are stored and are ineffective.

Delayed hatching could be due to: (1) reduced rate of embryonic development as a result of deficient maternal hormone, or (2) ineffective functioning of the chick thyroid during the last half of incubation.

REFERENCES

ABERLE, S. D., AND W. LANDAUER: *Anat. Rec.* 62: 331. 1935.
BATES, R. W., O. RIDDLE AND E. L. LAHR: *Endocrinology.* 29: 492. 1941.
FUGO, N.: *J. Exper. Zool.* 85: 271. 1940.
GROSSOWICZ, N.: *Proc. Soc. Exper. Biol. & Med.* 63: 151. 1946.
HOFFMANN, E., AND R. S. WHEELER: *Poultry Sci.* 27: 609. 1948.
HOLLANDER, W. F., AND O. RIDDLE: *Poultry Sci.* 25: 20. 1946.
HOPKINS, M. L.: *J. Morphol.* 58: 585. 1935.
LANDAUER, W.: *Biol. Symp.* 6: 127. 1942.
MCCARTNEY, M. G., AND C. S. SHAFFNER: (Univ. of Maryland, College Park). Paper presented at 37th annual meeting of Poultry Sci. Assn., June 1948.
MORENG, R. E., AND C. S. SHAFFNER: (Univ. of Maryland, College Park). Paper presented at 37th annual meeting of Poultry Sci. Assn., June 1948.
RIDDLE, O.: *Carnegie Institution of Washington Yearbook.* 25: 55. 1930.
SNEDECOR, G. W.: Statistical Methods, Iowa State College Press, Ames, Iowa, 1946.
SUN, T. P.: *Physiol. Zool.* 5: 384. 1933.
TURNER, C. W., M. R. IRWIN AND E. P. REINEKE: *Poultry Sci.* 24: 171. 1945.
UHLENHUTH, E. AND A. EBELING: *Anat. Rec.* 29: 398. 1925.
UKITA, T.: *Acta scholae med. univ. imp. in Kioto.* 3: 287. 1919.
WHEELER, R. S., AND E. HOFFMANN: *Endocrinology.* 42: 326. 1948a.
WHEELER, R. S., AND E. HOFFMANN: Paper presented at 9th annual meeting of Assn. of Southeastern Biologists, April 1948b.
WHEELER, R. S., AND E. HOFFMANN: Unpublished data. 1948c.

EFFECT OF ADRENALECTOMY AND ADRENAL CORTICAL HORMONE UPON THE FORMATION OF ANTIBODIES

JONATHAN S. THATCHER, BENJAMIN C. HOUGHTON AND CAROLYN H. ZIEGLER

From the Departments of Physiology and Medicine, The Ohio State University

COLUMBUS, OHIO

ONE OF THE striking characteristics of the adrenalectomized animal and of the Addisonian patient is a well-known susceptibility to infection.

Numerous attempts to study the effect of adrenal insufficiency upon the development of immunity has resulted in conflicting data. Interest in the relation of the adrenal cortical hormones to the development and release of antibody globulin in experimental animals has been revived in recent years by the work of Dougherty, White and associates (1945, 1946). These authors have noted an enhancement of immune titers in animals treated with cortical hormones or adrenotrophic fractions of the hypophysis during the period of immunization. Furthermore, animals previously treated with antigens, and in whom maximum titers had been attained, exhibited a remarkable augmentation of such titers following single injections of adrenal cortical extract or adrenotrophic hypophyseal extracts. This augmentation persisted for a period of 12 hours before returning to preinjection levels. The anamnestic effect exceeded the increase in immune titers induced by non-specific protein and specific antigen administration. Concurrent reduction in total lymphatic tissue, coupled with the observation that lymphocytes of immunized animals may yield immune globulin, led these authors to conclude that the adrenal hormones effected the dissolution of lymphocytes, thus releasing antibody to the constituent proteins of plasma. The hypothesis is further supported by two observations: (1) that adrenotrophic hormone fails to affect the titratable antibody in adrenalectomized animals; and (2) that desoxycorticosterone alone will neither augment initial titers nor produce the anamnestic effect after titers have been attained.

The present study was designed to yield information on three pertinent questions: (1) the capacity of the adrenalectomized animal to develop and maintain antibodies; (2) the effect of adrenal hormone in the release and/or distribution of antibody protein; (3) the relation-

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ship of the peripheral circulating lymphocyte values to the degree of attained immunity.

METHODS

Eleven normal adult cats and 3 normal adult rabbits were employed in these experiments. The animals were acclimatized to the laboratory for a period of at least 6 weeks prior to the experimental period.

The cats received a diet of 100 gm. of "Puss in Boots" cat food and 50 cc. of whole milk daily. The rabbits were fed Purina rabbit pellets with supplements of fresh lettuce every other day. All animals received water ad libitum. Bilateral adrenalectomy was performed in two stages, the operations being separated by a 2-week interval.

The animals were treated with whole adrenal cortical extract for a 10-day recovery period following the second operation. They were then maintained on 1 mg. daily of desoxycorticosterone acetate (DCA) in oil.

The antigen employed was a 10% suspension of fresh washed sheep erythrocytes in normal saline.

For the purpose of immunization, 2 series of antigen injections were administered intravenously. Each series consisted of 5 cc., 3 cc. and 3 cc. of antigen given on consecutive days. One day was allowed to elapse between series.

The hemolysin titration was carried out by the doubling dilution method, employing a 2% suspension of fresh washed sheep erythrocytes as antigen. Intermediate dilutions were made where finer determinations were necessary. Two units of standardized complement derived from pooled fresh guinea pig sera were used in the hemolysin titrations. All dilutions, in separate tubes, were incubated for one-half hour in a water bath at 37.5° C., after which the titers were read. Titers were reread after 12 hours of refrigeration. The highest dilution of antisera producing complete hemolysis of the sheep red blood cells was taken as the end-point of these determinations. Blood counts, including evaluation of RBC, WBC, determination of hemoglobin by the photolometer method, and differential counts by the supravital technique, were done at weekly intervals. The serum sodium levels were determined by the method of Butler and Tuthill (1931).

EXPERIMENTAL

Comparison of ability of normal and adrenalectomized cats maintained with desoxycorticosterone to form antibodies. Five cats were adrenalectomized and allowed to recover as described above. In view of the observation of Dougherty, Chase and White (1945) that desoxycorticosterone failed to influence the levels of circulating antibody, these animals were maintained following recovery with 1 mg. of DCA daily throughout the experimental period. With this dosage of DCA the plasma sodium values were maintained at normal levels. Seven days were allowed to elapse following initiation of DCA therapy before immunization was started, in order to allow establishment of an adequate equilibrium. These animals, with 6 intact control cats, were then immunized with a 10% suspension of washed sheep erythrocytes in 2 series as described above. All experimental animals survived this

immunization procedure in good condition. Marked anaphylactic reactions were observed in both the normal and the adrenalectomized cats during the second series of antigen injections. These reactions were characterized by: (1) dilatation of the pupils (2) nystagmus (3) salivation (4) marked slowing and diminished strength of pulse (5) initial acceleration followed by marked slowing of respiration (6) apprehensive cry (7) relaxation of sphincters and mass peristalsis.

Blood was drawn daily following the last administration of antigen for the determination of developing immune titers. Maximum titers were attained on the fifth day following the last injection of antigen.

TABLE 1

Group	Procedure	Animal	Maximum hemolysin titer*
I	Normal Intact	Cat *ME*	1:320
		Cat *NE*	1:160
		Cat *NI*	1:160
		Cat *NG*	1:80
		Cat *NK*	1:40
		Cat *NL*	1:40
		Average	1:133
II	Adrenalectomized (DOCA 1 mg. daily)	Cat *NN*	1:320
		Cat *MN*	1:160
		Cat *NF*	1:40
		Cat *NJ*	1:40
		Cat *NC*	1:40
		Average	1:120

* Highest titer developed during the experimental period.

As can be seen in Table 1, the titers attained by adrenalectomized DCA-maintained cats were equal to the highest titer exhibited by the control group. There is no significant difference in the range of titers shown by either group.

Following termination of the entire experimental period, DCA was withheld and all adrenalectomized animals died in acute adrenal insufficiency.

The variation in the leucocyte levels of the normal and adrenalectomized cats throughout the experimental period is illustrated in Figures 1 and 2. Although the fluctuations in all leucocyte values were great throughout the period of observation, neither these nor the absolute values of lymphocytes seemed to be related to the function of the adrenal gland nor to the degree of immunity attained by the animals.

The influence of whole adrenal extract upon the release or distribution of preformed antibodies in intact and adrenalectomized animals. Having established the capacity of the adrenalectomized animal to form antibodies, it was of interest to determine the effect of whole adrenal

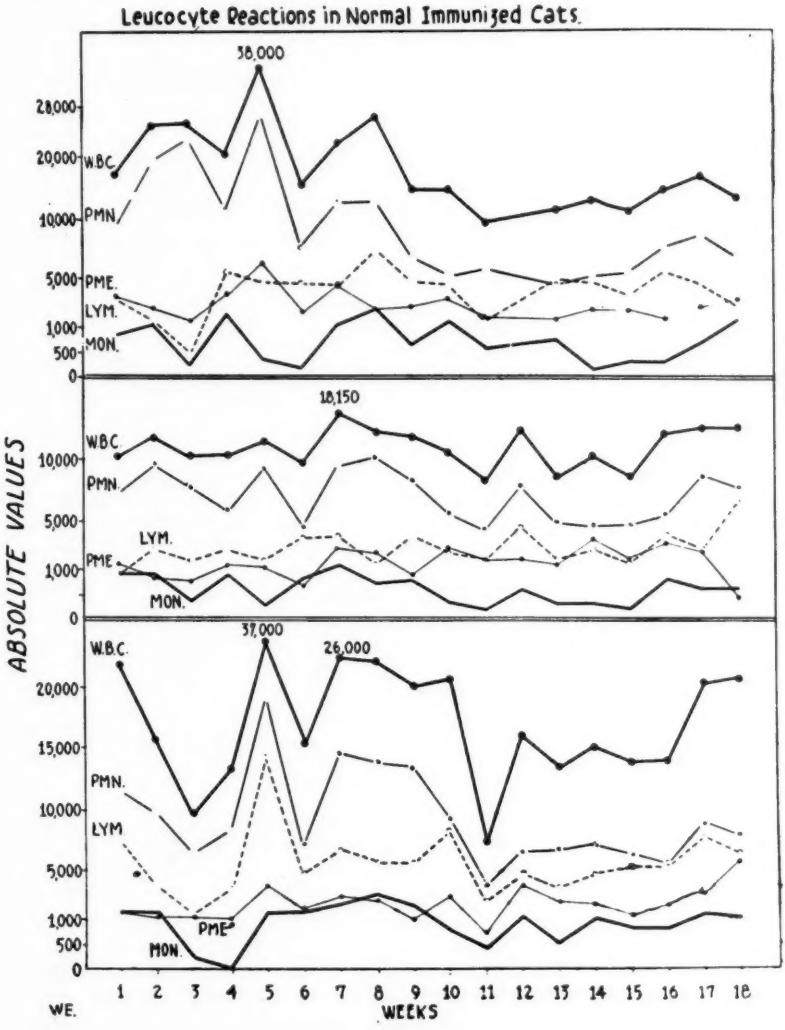

FIG. 1

cortical extract upon the circulating level of these specific immune bodies.

For these experiments, the same 2 groups of animals described above were employed. A single 10 cc. dose of whole adrenal extract (O.S.U.[1]) was administered both by the intravenous and subcutaneous

[1] This whole adrenal extract was prepared from whole beef adrenal glands according to the method described by Thatcher and Hartman (1946). Each cc. of extract represented 130 grams of beef adrenal gland tissue.

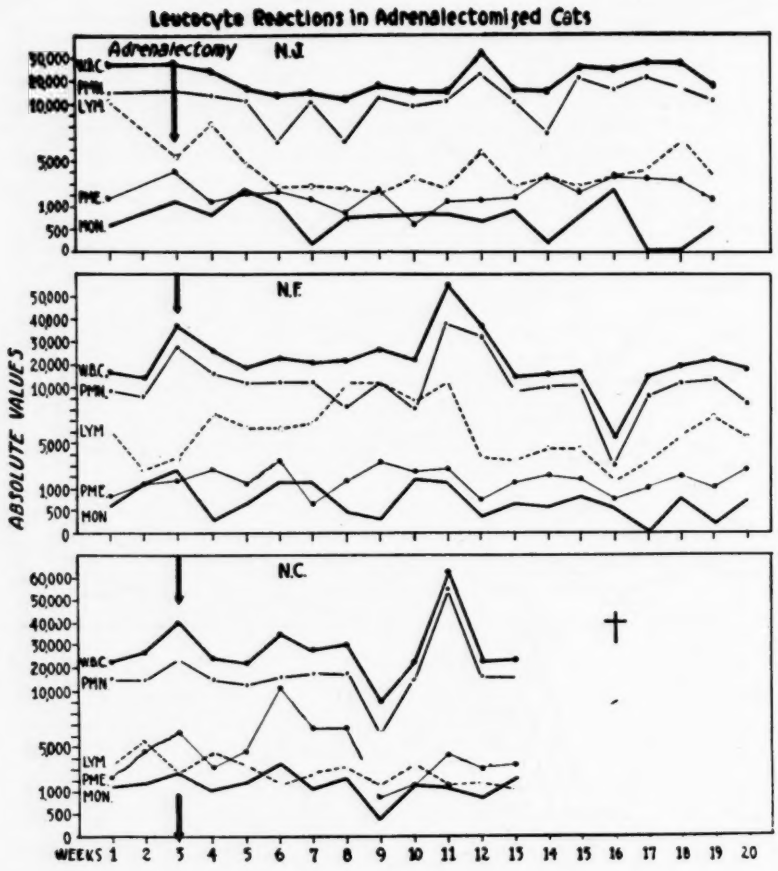

FIG. 2

routes following the establishment of baseline titers in both adrenalectomized and control groups. Blood for the determination of antibody levels was drawn at 3-hour intervals following administration of the extract. As can be seen from Table 2, the administration of adrenal extract, whether given intravenously or subcutaneously, resulted in no significant change in the level of circulating antibody in either adrenalectomized or normal cats.

The results obtained in these experiments were contrary to those reported by other workers using rabbits (Dougherty, Chase and White, 1945). Therefore, 3 normal adult rabbits were selected as additional controls and were immunized as described above, employing

TABLE 2

Group	Treatment	Animal	Hemolysin Titers				
			Base-line	3 hours	6 hours	9 hours	12 hours
I (Normal)	10 cc. ACE (OSU)	ME*	1:320	1:320	1:320	1:320	—
		NE	1:320	1:320	1:320	1:160	—
		NI	1:80	1:80	1:80	1:80	—
		NG	1:80	1:80	1:80	1:40	—
		NK*	1:40	1:40	1:40	1:40	—
		NL*	1:40	1:20	1:40	1:20	—
	10 cc. ACE (Wilson)	ME	0	0	0	0	0
		NE	1:10	1:10	1:10	1:10	1:10
		NG	0	0	0	0	0
II (Adrenalectomized) (DOCA 1 mg. daily)	10 cc. ACE (OSU)	NF	1:20	1:20	1:10	1:20	1:20
		NJ	1:10	1:10	1:10	1:10	1:10
		NC	1:20	1:10	1:20	1:10	1:10
	10 cc. ACE (Upjohn)	NN	1:160	1:160	1:160	1:80	1:80
		MN	1:80	1:80	1:80	1:80	1:80

* Extract administered subcutaneously.

3 series of injections of sheep erythrocytes. Although the titers attained were much higher than in cats, the administration of whole adrenal extract (O.S.U.) failed to produce any change in circulating antibody level (Table 3).

The possibility that differences in the extract employed by us and that used by other workers could account for the discrepancy in these results had to be considered. Hence, these experiments were repeated in the same normal immunized cats and rabbits by administering 10 cc. of Wilson commercial whole adrenal extract intravenously. Although at the time this experiment was undertaken, the hemolysin titers had fallen to low levels, no augmentation was observed (Tables 2 and 3). Subsequently, 2 of the adrenalectomized cats (MN, NN) were similarly treated, using the commercial extract prepared by The Upjohn Company. This preparation also failed to yield an anamnestic response (Table 2).

TABLE 3

Group	Animals	Treatment	Hemolysin Titers					
			Maximum*	Base-line	3 hours	6 hours	9 hours	12 hours
III	Rab. A	10 cc. ACE (OSU)	1:6000	1:1000	1:1000	1:1000	1:1000	1:1000
	Rab. B		1:8000	1:1000	1:1000	1:1000	1:1000	1:1000
	Rab. 2		1:4000	1:1000	1:1000	1:1000	1:1000	1:1000
	Rab. A	10 cc. ACE (Wilson)		1:200	1:200	1:200	1:200	1:200
	Rab. B			1:400	1:200	1:200	1:50	1:100
	Rab. 2			1:50	1:50	1:50	1:50	1:50

* Highest titer developed.

DISCUSSION

The ability of the animal organism to yield antibodies following adrenalectomy has been the subject of considerable controversy. The literature reveals that in some instances titers were increased, in others decreased (Jaffe and Marine, 1924; Blanchard, 1931). Perla and Marmorston-Gottesman (1929) report temporary augmentation, with subsequent reduction. More recently, Murphy and Sturm have reported marked increases in antibody globulin in adrenalectomized rabbits (1947). Chase, White and Dougherty (1946) have pointed out the difficulties involved in the maintenance of adrenalectomized animals for such study and suggested that accessory cortical tissue or incomplete removal of glands could account for the variations reported. It has been the experience of many workers, however, that cats may be more readily rendered cortically insufficient than most other species of laboratory animals.

The results here indicate that adrenalectomized cats maintained on DCA are capable of forming antibodies in magnitude equal to those of normal intact cats. These findings are in agreement with the results obtained by Eisen and associates using similarly treated rats (1947). It may be argued that since DCA maintains a certain degree of physiological equilibrium it may also sustain the mechanism for antibody formation. However, Dougherty, Chase and White (1945) have emphasized that desoxycorticosterone has no effect upon the antibody levels of intact immunized animals nor does it elicit an anamnestic response in such animals. The fact that all of the adrenalectomized cats in our series died in acute adrenal insufficiency following withdrawal of DCA therapy attests the completeness of their adrenal deprivation. In contrast to the findings of Chase, White and Dougherty (1946), all attempts to produce an anamnestic response with adrenal cortical extract failed. The failure of whole adrenal extract to produce an anamnestic response in the adrenalectomized animals is particularly significant, for if the levels of antibody titers are to be explained by the rate of lymphocyte lysis (White and Dougherty, 1946), whole adrenal extract, acting upon the hyperplastic lymphoid tissue of adrenalectomized animals, would be expected to raise the titers to relatively high levels.

Our inability to reproduce the anamnestic response cannot be attributed to either a species difference or a difference in the type of extract employed, since both cats and rabbits were subjected to trials with 3 different potent extract preparations (O.S.U., Upjohn and Wilson).

Hematologic studies carried out concurrently on normal immunized cats and rabbits and adrenalectomized cats revealed wide variations in total lymphocyte levels which have no relation to the presence or absence of the adrenal gland nor to the capacity of the animal to form or maintain specific hemolysin titers.

SUMMARY

Adrenalectomized cats maintained with DCA demonstrated an ability to produce antibodies at levels comparable with those shown by normal intact control cats. Single large doses of adrenal cortical extract failed to alter titers of immune body when they were established. There was no demonstrable correlation between the levels of circulating lymphocytes and the antibody titers studied.

ACKNOWLEDGMENT

The authors wish to acknowledge the work of Mr. Adolph Ackerman, who carried out the hematologic studies on the experimental animals reported here.

REFERENCES

BLANCHARD, E. W.: *Physiol. Zool.* 4: 302. 1931.
BUTLER, A. M., AND E. J. TUTHILL: *J. Biol. Chem.* 93: 171. 1931.
CHASE, J. H., A. WHITE AND T. F. DOUGHERTY: *J. Immunol.* 52: 101. 1946.
DOUGHERTY, T. F., J. H. CHASE AND A. WHITE: *Proc. Soc. Exper. Biol. & Med.* 58: 135. 1945.
EISEN, H. N., M. M. MAYER, D. H. MOORE, R. R. TARR AND H. C. STOERK: *Proc. Soc. Exper. Biol. & Med.* 65: 301. 1947.
JAFFE, H. L., AND D. MARINE: *J. Inf. Dis.* 35: 334. 1924.
MURPHY, J. B., AND E. STURM: *Proc. Soc. Exper. Biol. & Med.* 66: 303. 1947.
PERLA, D., AND J. MARMORSTON-GOTTESMAN: *J. Exper. Med.* 50: 87. 1929.
THATCHER, J. S., AND F. A. HARTMAN: *Arch. Biochem.* 10: 195. 1946.
WHITE, A., AND T. F. DOUGHERTY: *Endocrinology.* 36: 207. 1945.
WHITE, A., AND T. F. DOUGHERTY: *Ann. New York Acad. Sc.* XLVI. Art. 8: 859. Sept. 1946.

NOTES AND COMMENTS

ATTEMPTS AT PHARMACOLOGIC BLOCKADE OF THE SECRETION OF ADRENOCORTICOTROPHIN

Recently, Long (1947) has reviewed the evidence in support of the hypothesis that adrenocorticotrophin is released from the hypophysis in response either to circulating epinephrine or to adrenergic nerve stimulation. Markee and others (1947) have advanced a similar theory in connection with post-coital ovulation in rabbits. Sawyer et al. (1947) reported that the pretreatment of rabbits with Dibenamine prevented ovulation from occurring following the direct instillation of epinephrine into the hypophysis.

In view of the current interest in drugs which block adrenergic responses, it was considered pertinent to test the effects of large doses of Dibenamine and tetraethylammonium chloride on the ability of the pituitary-adrenal axis to respond to a standard test stimulus.

In the first experiment it was found that the intraperitoneal injection of 25 mg./kg. of body weight of Dibenamine itself produced a 35% fall in adrenal ascorbic acid concentration (method of Roe and Kuether, 1943), which then rose steadily to normal levels within ten hours. However, since Nickerson and Goodman (1947) have shown that Dibenamine exerts its

TABLE 1. ADRENAL ASCORBIC ACID CONCENTRATIONS IN "DIBENAMIZED," COLD-EXPOSED AND CONTROL ANIMALS ONE HOUR AFTER INTRAPERITONEAL INJECTION WITH CCl_4 (3ml./kg.) (Means and their standard errors are given for each group)

Group	No of Rats	Description	Body Wt. Grams	Adrenal Wt. mg/100 gm. rat	Adrenal Ascorbic Acid mg./100 gm. adrenals	Adrenal Ascorbic Acid percent decrease
A	6	Controls	170 ± 6.5	15.3 ± 0.78	429.5 ± 22.7	
B	6	CCl_4	167 ± 3.8	15.0 ± 1.51	287.5 ± 15.1	33
C	6	Dibenamine	169 ± 6.2	14.8 ± 0.85	450.6 ± 18.4	
D	5	Dibenamine and CCl_4	165 ± 7.5	12.8 ± 1.10	292.8 ± 7.6	35
E	6	Exposed 7°C.	168 ± 5.0	14.9 ± 0.54	468.5 ± 13.0	
F	5	Exposed 7°C. and CCl_4	164 ± 7.5	16.2 ± 1.46	324.6 ± 22.0	31

blocking action over a period of days, the feasibility of testing ACTH response 24 hours after injection of the drug was considered. Accordingly, in a second experiment six groups of animals were studied as indicated in Table 1. The standard stimulus was 3 ml. of CCl_4 per kg., intraperitoneally, and animals were sacrificed (pentobarbital anesthesia) 60 minutes following this injection. Rats in groups C and D were injected with Dibenamine (25 mg. per kg. of body weight) 24 hours before the carbon tetrachloride test. Since the animals given Dibenamine were known to have had a transitory activation of the ACTH mechanism on the day before the test, another set of rats were included as controls. These were exposed to a temperature of 7° C.

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for three hours one day before carbon tetrachloride injection. The experimental results, summarized in Table 1, show that under the conditions of these experiments Dibenamine does not inhibit the response of the pituitary-adrenal axis to a standard stress.

It should be pointed out that, although a block of the ACTH response in the "Dibenamized" animals would have constituted strong presumptive evidence in favor of the physiologic participation of some epinephrine-like substance in the response, the failure to produce a block does not rule out the possibility that epinephrine may be involved in the release of pituitary ACTH. Nickerson and Goodman (1947) have described certain responses to epinephrine which are not blocked in the Dibenamine-treated animal.

TABLE 2. ADRENAL ASCORBIC ACID RESPONSE TO INTRAPERITONEALLY ADMINISTERED CCl_4 IN TETRAETHYLAMMONIUM-PRIMED RATS AND CONTROLS
(Means and their standard errors are given for each group)

Group	No. of Rats	Description	Body Wt. Grams	Adrenal Wt. mg./100 gm. rat	Adrenal Ascorbic Acid mg./100 gm. Adrenals	Adrenal Ascorbic Acid percent Decrease
A	5	Controls	161 ± 3.9	15.9 ± 1.0	482.6 ± 19.1	
C	6	CCl_4	161 ± 6.8	13.5 ± 0.8	333.8 ± 18.7	31
B	6	TEA	163 ± 6.1	14.3 ± 1.0	448.1 ± 14.8	
D	6	TEA and CCl_4	167 ± 5.8	12.9 ± 0.7	333.5 ± 13.4	26

In a third experiment the ACTH response was tested in animals which had been injected intraperitoneally with 10 mg./kg. of tetraethylammonium chloride (Acheson and Moe, 1946) five minutes before the injection of CCl_4. Although the injection of tetraethylammonium itself produces no significant fall in adrenal ascorbic acid detectable after 65 minutes, it did not prevent a fall of 26% in animals that received CCl_4 intraperitoneally (See Table 2). Thus, under the conditions of this experiment the chemo-receptor function of the anterior pituitary in the presence of a standard stimulus was unimpaired by pretreatment with tetraethylammonium ion. However, it can not be stated from available evidence that the autonomic ganglia of the tetraethylammonium-injected rats were completely blocked for the duration of the test period.

These results, then, neither support nor invalidate the epinephrine hypothesis of ACTH stimulation. It is relevant to remark, however, that the many patients who have received Dibenamine and/or tetraethylammonium probably have not at the same time experienced a transitory adrenal insufficiency of pharmacologic origin.

JAY TEPPERMAN AND JEAN S. BOGARDUS

From the Department of Pharmacology,
Syracuse University College of Medicine,
Syracuse, N. Y.

(Aided by a grant from the Hendricks Research Fund of the Syracuse College of Medicine.)

REFERENCES

ACHESON, G. H., AND G. K. MOE: *J. Pharmacol. & Exper. Therap.* 87: 220. 1946.
LONG, C. N. H.: *Federation Proc.* 6: 461. 1947.
MARKEE, J. E., C. H. SAWYER AND W. H. HOLLINSHEAD: *Anat. Rec.* 97: 398. 1947.
NICKERSON, M., AND L. GOODMAN: *J. Pharmacol & Exper. Therap.* 89: 167. 1947.
ROE, J. H., AND C. A. KUETHER: *J. Biol. Chem.* 147: 399. 1943.
SAWYER, C. H., J. E. MARKEE AND H. HOLLINSHEAD: *Anat. Rec.* 99: 597. 1947.

ERRATA NOTICE

In the text of the paper by Ingle and Nezamis, entitled "The Work Performance of Adrenalectomized Rats Given Continuous Intravenous Infusions of Glucose" which appeared in Endocrinology 43: 261-271 (October) 1948, it was erroneously stated that adrenal cortex extract (ACE) was administered in amounts of 20 cc. per 24 hours per rat. The correct amount, as shown in Figures 1 and 2, was 5 cc. diluted to a 20 cc. total volume per 24 hours per rat.

ASSOCIATION NOTICE

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

NOTES ON THE THIRTIETH ANNUAL MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting was held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

Forty-seven papers were presented and forty-four papers were read by title. Total registration was 458, equally divided between members and non-members. Two hundred, twenty-two were present at the dinner at which the presidential address was given by Doctor C. N. H. Long.

Actions of general interest taken by the Council are as follows:

(1) It was the decision of the Council to hold a Postgraduate Assembly in Oklahoma City, February 21–26, 1949, similar to the one which was so well received in Los Angeles in 1948.

(2) The Council approved the indexing and publication of ENDOCRINOLOGY, Volumes 1 to 40, inclusive. These will be published by Mr. Charles C Thomas, in two editions, one including the indices of Volumes 1 to 25, and the other, Volumes 26 to 40.

(3) It was the Council's decision to publish the transactions of the American Goitre Association.

(4) The Council accepted with much regret the resignation of Doctor Earl T. Engle, Chairman of the Publications Committee, and appointed Doctor Warren O. Nelson to this office. Appreciation was expressed to Doctor Engle for his work as chairman of this committee.

(5) The Committee on Registry of Endocrine Pathology was re-appointed to negotiate with the Scientific Director of the American Registry of Pathology regarding the formation of an Endocrine Registry at the Army Institute of Pathology. A sum not to exceed $750.00 was appropriated for necessary expense for one year.

(6) It was voted to appropriate $250.00 for 1949 to the National Society for Medical Research.

(7) Due to increasing cost of labor, paper and all printing materials, and the enlargement of the Journals, the dues were increased to $11.00 per year which includes subscription to either of the Journals, with a combination offer of $16.00 for both Journals.

(8) The Council voted that the thirty-first Annual Meeting be held June 3 and 4, 1949, in Atlantic City, New Jersey.

(9) A list of the 1948–49 Officers, Council and Committees follows:

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ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

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